(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 April 2001 (26.04.2001)

PCT

(10) International Publication Number
WO 01/29036 A2

(51) International Patent Classification[7]: C07D 471/00

(21) International Application Number: PCT/GB00/04064

(22) International Filing Date: 20 October 2000 (20.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9924962.5 21 October 1999 (21.10.1999) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/29036 A2

(54) Title: ALLOSTERIC SITES ON MUSCARINIC RECEPTORS

(57) Abstract: An allosteric site on muscarinic receptors is disclosed, together with its use for screening for compounds capable of modulating the binding of a primary ligand such as acetylcholine to the receptor. The site is characterised herein a series of indolocarbazoles represented by formula (1) and a series of related compounds represented by formula (2). These compounds are capable of binding to the allosteric site to modulate the binding of a primary ligand to the receptors, showing positive, negative and neutral cooperativity and selectivity for muscarinic receptor subtypes.

Allosteric Sites on Muscarinic Receptors

Field of the Invention

The present invention relates to muscarinic receptors, and in particular to compounds which are capable of binding to an allosteric site on a muscarinic receptor and modulating the binding of a primary ligand such as acetylcholine to the receptor. The present invention further relates to methods for aiding in the identification of compounds which bind to the allosteric site and their use in methods of medical treatment.

Background of the Invention

The five muscarinic receptors subtypes are designated M_1-M_5 and all are activated by the binding of acetylcholine (ACh). These receptor subtypes are widely distributed in the central nervous system and in the periphery where they mediate a number of important physiological functions. As a consequence these receptors are a therapeutic target for the treatment of a variety of conditions and potential therapeutic agents are both agonists and antagonists. In the treatment of many conditions it has been thought to be important that the therapeutic agents have a selective action on one or a limited number of subtypes. However, there remains a problem in the art that the muscarinic receptor subtypes are structurally very similar as a consequence of the identity of amino acids in the regions of sequence that are considered to constitute the ACh binding site, i.e. the site of binding of agonists and competitive antagonists. Therefore, it has not been possible to synthesize highly selective muscarinic antagonists and no directly acting muscarinic agonists of any substantial selectivity exist (Caulfield and Birdsall, 1998). A further problem is that synthetic exogenously applied agonists chronically stimulate receptors and this can

result in desensitization and downregulation of the receptor function as well as losing any information content of the pulsatile endogenous ACh signalling mechanism.

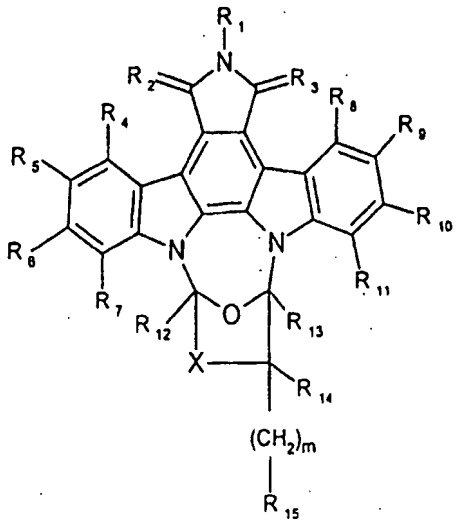
However, in addition to the primary site on these receptors at which agonists and competitive antagonists bind, muscarinic receptors are known to also contain an allosteric site. Compounds binding at the allosteric site mediate the binding of the ligands to the primary binding site (GB 2 292 685 A and WO 96/03377). Thus, it is possible that compounds binding at the allosteric site may overcome some of these problems involved in selective modulation of muscarinic receptor subtypes. By way of example, it is known that when brucine and some of its N-substituted analogues bind at the allosteric site, they modulate the response of muscarinic receptors to the primary ligand acetylcholine (ACh) or N-methylscopolamine (NMS), a competitive antagonist of ACh. The modulation caused by compounds binding at the allosteric site can be positive, negative or neutral. A compound which has neutral cooperativity with ACh at one muscarinic receptor subtype binds to the receptor but has no action at any concentration. In contrast, if the same ligand has positive or negative cooperativity at another subtype it has an action at that subtype which is totally selective. This form of selectivity based on cooperativity can be termed 'absolute subtype selectivity'. Thus, the allosteric agents can modulate the interaction between the muscarinic receptor and the primary ligand.

Summary of the Invention

Broadly, the present invention relates to the finding that muscarinic receptors have a further allosteric site which is characterised herein using compound 1a, and a

series of related indolocarbazoles represented by formula 1, and compounds 2a and 2b, and a series of related compounds represented by formula 2. These compounds are capable of binding to the allosteric site to modulate the binding of a primary ligand to the receptors, showing positive, negative and neutral cooperativity and selectivity for muscarinic receptor subtypes.

Compounds represented by formula 1:



wherein:

R_1 is hydrogen, lower alkyl, aralkyl, iminoalkyl or an imino protecting group such as an acyl group $R_{19}CO$, where R_{19} is alkyl or aralkyl;

R_2 and R_3 are independently an oxygen or two hydrogen atoms;

R_4-R_{11} are independently selected from hydrogen or general aromatic substituents e.g. halo, nitro, cyano, lower alkyl, haloalkyl, alkoxy, hydroxy, aralkoxy;

R_{12} is H or lower alkyl;

X is CH_2,

$CH(OR_{16})$, where R_{16} is hydrogen, lower alkyl or an O-protecting group,

$CH(OR_{16})-CH(OR_{16})$,

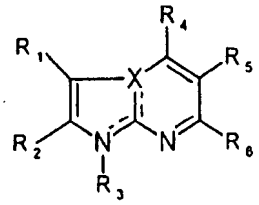
$CH(OR_{16})-CH(CH_2)_n-N(R_{17})_2$, where n is an integer between 0-5 and R_{17} is one or two of the following; alkyl, aralkyl;

R_{14} is hydrogen, OR_{16}, NHR_{17}, where R_{17} is alkyl, haloalkyl, aralkyl or an acyl group;

m is an integer between 0-8; and,

R_{15} is hydrogen, CO_2R_{18}, $CONHR_{18}$ or an isostere for an ester or substituted ester, where R_{18} is hydrogen, alkyl or substituted alkyl, aralkyl or substituted aralkyl.

Compounds represented by formula 2:



wherein:

X is N(H) or C(H);

R_1, R_2 are independently hydrogen, general aromatic substituents e.g. halo, nitro, cyano, lower alkyl, haloalkyl, alkoxy, hydroxy, aralkoxy;

R_3 is hydrogen, alkyl, iminoalkyl, or aralkyl;

R_1 and R_2 together and/or R_2 and R_3 together are a fused aromatic or heterocyclic system optionally with ring substituents;

R_4, R_5, R_6 are normal aromatic substituents;

or R_5, R_6 together are a fused alicyclic system with 1-5 rings including steroid ring systems, preferably with substituents at either or both 17α and 17β positions, e.g. hydroxy, alkoxy, aralkoxy, alkyl, alkenyl, or alkynyl, and R_4 is a substituent compatible with the

synthetic method;

or R_5, R_6 together are a fused alicyclic system with 1-5 rings and R_4 is hydrogen or an alkyl group;

or R_4, R_5, R_6 are part of a fused alicyclic system;

and a dotted line indicated either the presence or the absence of a bond.

Preferably, the above generally defined substituents are C_1-C_{10}, or in the case of lower alkyl substituents, C_1-C_6; and in either case, optionally including branching and/or halogen substitution.

The formulae of compounds 1a, 2a and 2b are shown on pages 54 to 56.

Accordingly, in a first aspect, the present invention provides a compound represented by formula 1 or 2 for use in a method of medical treatment.

In a further aspect, the present invention provides the use of a compound for the preparation of a medicament for the treatment of a condition mediated by the binding of a primary ligand to a muscarinic receptor, wherein the compound binds to an allosteric site of the muscarinic receptor which is capable of binding to compound 1a and/or 2a and thereby modulates the binding of the primary ligand to the muscarinic receptor. Preferred compounds include those represented by formula 1 or 2.

In a further aspect, the present invention provides a method of modulating the response of a muscarinic receptor to a primary ligand, the method comprising contacting the muscarinic receptor with a compound which binds to an allosteric site of the muscarinic receptor which is capable of binding to compound 1a and/or 2a and

which thereby modulates the binding of the primary ligand to the muscarinic receptor. This method may be carried out *in vitro*, e.g. as part of a screening method or to otherwise activate or modulate the response of the receptor, or *in vivo*, e.g. in the treatment of a patient suffering from one of the conditions described herein.

The primary ligand binding to the muscarinic receptor may be an agonist or an antagonist of the receptor's biological activity. Examples of primary ligands include acetylcholine (ACh) or N-methylscopolamine (NMS). Other primary ligands are well known to those skilled in the art.

The use of a compound which binds to this allosteric site, and in particular an allosterically acting compound which has a positive or negative cooperative effect on the binding of the primary ligand, can have the advantage of selectively modulating the natural function of a limited group of the muscarinic receptor subtypes, and more preferably only a single muscarinic receptor subtype. Thus, the invention helps to solve the problem of selectively activating the function of specific muscarinic receptor subtypes in a way which is difficult to achieve using a primary ligand which binds to multiple receptor subtypes, and opens up the possibility of therapeutic treatment based on this selectivity. Examples of these conditions are discussed below. While it is generally preferred that the binding of the allosteric compound to the muscarinic receptor enhances the binding of the primary ligand (i.e. shows positive cooperativity), compounds which decrease the binding of the primary ligand (i.e. act antagonistically or show negative cooperativity) can also have therapeutic potential. Such compounds have the property of not

blocking 100% of the receptor response when they bind to the allosteric site of the receptor (as competitive antagonists do). These compounds can be used in the treatment of conditions including Alzheimer's disease, motion sickness, depression, bronchitis, gastric and duodenal ulcers, non ulcer dyspepsia, urinary bladder incontinence and retention, sinus bradycardia, Parkinson's disease, incontinence, asthma, chronic obstructive pulmonary disease, irritable bowel syndrome, excessive vagal drive, as a preanaesthetic, for cardiac pacemaker regulation, or for the regulation of sleep.

The allosteric site defined herein is distinct from the 'common allosteric site' disclosed in the prior art (e.g. GB 2 292 685 A) which binds to gallamine, strychnine, brucine and N-substituted brucine analogues. In contrast, the present inventors have found a new allosteric site which binds compound 1a and a series of related indolocarbazoles having formula 1 and compound 2a and a series of related compounds having formula 2.

Preferably, the muscarinic receptor is selected from the M_1, M_2, M_3, M_4 or M_5 receptors known in the art. The receptors may be human or an appropriate animal homologue (rat, mouse etc). The generation of transfected cell lines stably or transiently expressing one or more of the M_1-M_5 receptor genes from any given species is well in the art and relevant references are cited, for example, in the reviews: Hulme et al, 1990 and Caulfield and Birdsall, 1998.

Exemplary compounds which are capable of modulating the binding of a primary ligand to a muscarinic receptor by interaction with the allosteric site described for the first time herein include compounds 1a, 2a and 2b.

In a further aspect, the present invention provides a method for aiding in the identification of compounds capable of modulating the binding of a primary ligand to a muscarinic receptor by binding to an allosteric site of the muscarinic receptor which is capable of binding to compound 1a and/or compound 2a, the method comprising:

(a) contacting the muscarinic receptor and the primary ligand with one or more concentrations of a candidate compound; and,

(b) determining whether the candidate compound modulates the binding of a primary ligand to the muscarinic receptor by binding to the allosteric site of the receptor which is capable of binding compound 1a and/or compound 2a.

The method may comprise the further step of selecting a candidate compound which modulates the binding of the primary ligand to the muscarinic receptor.

In a further aspect, the present invention provides the use of an allosteric site of a muscarinic receptor which is capable of binding to compound 1a and/or 2a in screening for compounds which are capable of modulating the binding of a primary ligand to a muscarinic receptor by binding to the allosteric site.

The modulation of the binding of the primary ligand may be achieved by a number of mechanisms. The allosteric compound may have a positive or negative cooperative effect at one or more of the muscarinic receptor subtypes, and preferably no effect (neutral cooperativity) at other receptor subtypes. Alternatively or additionally, the binding of the allosteric compound may affect the binding of an agent acting at a different allosteric site such as the common allosteric site which

binds brucine.

Preferably, candidate allosteric compounds are selected if they have a positive or negative cooperative effect on the binding of the primary ligand. Thus, in preferred embodiments, step (b) involves determining whether the candidate compounds bind to the allosteric site and additionally determining how the binding modulates the action of the primary ligand at its binding site. This can be carried out using the assays described herein including equilibrium and/or kinetic binding assays and/or functional assays.

General assays which are suitable or can be adapted for use in the present invention are described in Lazareno and Birdsall (1995), Detection, quantitation and verification of allosteric interactions of agents with labelled and unlabelled ligands at G-protein-coupled receptors: Interactions of strychnine and acetylcholine at muscarinic receptors. Mol. Pharmacol. 48:362-378; Lazareno et al (1998), 'Subtype selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: radioligand binding studies. Mol. Pharmol. 53:573-589; Birdsall et al (1999), Subtype selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: functional studies. Mol. Pharmacol. 55:778-786.

Examples of specific assays which can be employed are described in the 'Materials and Methods' section below.

Firstly, the candidate compounds are selected as being allosteric using the criteria defined in the above general and specific assays. The compounds are further

selected as binding to the novel site, e.g. the site which binds compound 2a, using specific kinetic and equilibrium assays. For example, any allosteric compound which is not competitive with the binding of brucine, gallamine or strychnine and is competitive with the binding of 2a to the novel allosteric site is a candidate compound for further investigation or development as a therapeutic. These methods are useful for finding compounds which are positively, negatively or neutrally cooperative with the binding of the primary ligand.

In preferred embodiments, the screening is carried out using a receptor, one or more concentrations of a candidate allosteric ligand (possibly including an assay carried out in the absence of the candidate ligand by way of control) and one or more primary ligands, in the presence or absence of another allosteric ligand. Preferably, the primary ligand employed in this screening method is acetylcholine (ACh) and/or N-methylscopolamine (NMS), although other suitable primary ligands are well known to those skilled in the art. In one embodiment, the method involves as assay in which the binding of a candidate compound to the allosteric site is determined in using labelled NMS in the absence or presence of one or more concentrations of ACh. Alternatively or additionally, the binding of a candidate compound to the allosteric site is determined using labelled NMS in assays which determine the NMS dissociation rate constant in the presence and absence of one or more concentrations of the candidate compound. Another preferred assay format is an assay of the effects of one or more concentrations of an allosteric ligand on the acceleration of the dissociation rate of NMS from muscarinic receptors produced by 2a (described in Part II below). A further assay is to quantitate the effects of

a test compound, which has been demonstrated in the general assays to be allosteric, on the equilibrium allosteric effects of ligands which are known to bind one or other of the two allosteric sites described in Part I, Figure 5).

In these methods, the candidate compound may be selected if it enhances the binding of the primary ligand to the muscarinic receptor (otherwise referred to as positive cooperativity). However, other compounds may be selected if they reduce the binding of the primary ligand to the muscarinic receptor (otherwise referred to as negative cooperativity). Candidate compounds having neutral cooperativity are selected if they bind to one or more of the muscarinic receptor subtypes but have no action on the equilibrium binding of a primary ligand at any concentration.

The allosteric site employed in the work described herein is capable of binding compounds 1a and/or 2a. In contrast to the first allosteric site disclosed in the prior art, the site described herein does not bind to brucine, gallamine and/or strychnine to any substantial extent at concentrations up to 10^{-4}M.

In a further aspect, the present invention provides a method which comprises, having identified a candidate compound by the above method, the further step of manufacturing the compound in bulk and/or formulating the compound as a pharmaceutical composition.

In a further aspect, the present invention provides the use of a compound as obtainable by the above method for the preparation of a medicament for the treatment of a conditions mediated by the binding of the primary ligand

to the muscarinic receptor. Examples of these conditions discussed below.

Embodiments of the invention will now be described by way of example and not limitation with reference to the accompanying drawings.

Brief Description of the Figures

Figure 1: Effect of staurosporine (1f) on the binding of ^{3}H-NMS (210 pM) at M_1 receptors in the absence and presence of 2.2 mM ACh, all in the presence of 0.2 mM GTP. The points are individual observations. The lines show the fit to Equation 2 (see Methods), which yielded a log affinity of 5.95 ± 0.06, a slope factor of 1.01 ± 0.05, cooperativity with ^{3}H-NMS of 1.51 ± 0.06, and cooperativity with ACh of 0.27 ± 0.03. The affinity ratio plots of these data are shown in Figure 2.

Figure 2: Affinity ratio plots of five indolocarbazoles (1a, 1b, 1e, 1f and 1i) at M_1-M_4 receptors. The points were derived from duplicate observations of ^{3}H-NMS binding in the absence and presence of ACh, as described in Methods. The parameter estimates pK (log affinity of the test agent for the free receptor), α_{NMS} (cooperativity with ^{3}H-NMS), and $ß_{ACh}$ (cooperativity with ACh) were derived from nonlinear regression analysis with Equations 1 or 2 as appropriate (see Methods). The parameter estimates from a number of similar assays are summarised in Table 1.

Figure 3: Effect of 1a,1b, 1e, 1f and 1i on the dissociation rate constant (k_{off}) of ^{3}H-NMS at M_1-M_4 receptors, expressed as a percent inhibition of the control k_{off}. The points are the mean and range/2 of duplicate observations. The lines show the fit to a

logistic function, as described in Methods. The parameter estimates from a number of similar assays are summarised in Table 2.

Figure 4: Effect of various concentrations of KT5720 (1a) on the inhibition of ^{3}H-NMS (50 pM) binding at M_1 receptors by ACh in a volume of 3 ml. The points are the mean and range/2 of duplicate observations. The lines show the fit to Equation 1 with the slope factor for KT5720 binding set to 1. The parameter estimates were: log affinity of KT5720 6.6 ± 0.1, cooperativity with ^{3}H-NMS 1.9 ± 0.1, cooperativity with ACh 1.6 ± 0.2. The inset shows affinity ratio plots derived from these parameters (see Methods). The -log IC_{50} values of ACh in the presence of increasing concentrations of KT5720, from independent logistic fits of the curves, were 5.28, 5.33, 5.40 and 5.42.

Figure 5: Inhibition by gallamine of ^{3}H-NMS binding at M_1 receptors in the presence of various concentrations of (A) KT5720 (1a) and (B) staurosporine (1f). The points are individual observations. The lines show the fit of the data to Equation 3, where the cooperativity estimates of gallamine with KT5720 and staurosporine were not significantly different from 1 and 0 respectively and were set to those values. The slope factors for gallamine, KT5720 and staurosporine were not different from 1 and were set at that value. From three such assays, similarly constrained, KT5720 had a log affinity of 6.22 ± 0.17 and cooperativity with ^{3}H-NMS of 2.39 ± 0.08. From three such assays, similarly constrained, staurosporine had a log affinity of 5.75 ± 0.11 and cooperativity with ^{3}H-NMS of 1.62 ± 0.13. From these six assays gallamine had a log affinity of 5.05 ± 0.05 and cooperativity with ^{3}H-NMS of 0.11 ± 0.01. The insets show

the effect of the test agent on the -log IC_{50} of gallamine, obtained from nonlinear regression analysis of the individual curves.

Figure 6: Effect of KT5720 (1a) on ^{3}H-NMS dissociation from M_1 receptors, alone and in the presence of other allosteric agents, measured at a single time point as described in Methods. The points show the mean and s.e.m of quadruplicate observations obtained in two assays, except for 10^{-4}M gallamine, and 3.10^{-5}M and 3.10^{-4}M brucine which show the mean and range/2 of duplicate observations. The lines in the top panel show the fits of the individual curves to a hyperbolic function, except for those in the presence of staurosporine. The estimates of the log EC_{50} of KT5720 derive from those fits. The top panel shows the data as % inhibition of the control k_{off} of ^{3}H-NMS. The lower panel shows shows Ef, the ^{3}H-NMS k_{off} values in the presence of KT5720 and a certain concentration of test agent (gallamine, brucine or staurosporine) as a fraction of the k_{off} values in the presence of that concentration of test agent alone.

Figure 7: Concentration-dependent effects of the compounds shown on pages 55 and 56 on the ^{3}H-NMS-occupied receptor. The percent inhibition of the dissociation rate constant of ^{3}H-NMS from M_1-M_4 receptors was measured at a single time point. The legends indicate the IC_{50} (or EC_{50}) values obtained using nonlinear regression analysis of these curves.

Figures 8 and 9: Concentration-dependent effects of active compounds on the equilibrium binding of ^{3}H-NMS and ACh. The effects are expressed as 'affinity ratios', i.e. the apparent affinity of the 'primary' ligand (^{3}H-NMS or ACh) in the presence of a particular concentration of

test agent divided by its apparent affinity in the absence of test agent. Affinity ratios were calculated as described in 'Methods'. Affinity ratios > 1 indicate positive cooperativity, affinity ratios < 1 indicate negative cooperativity, and affinity ratios of 1 with one primary ligand at concentrations of test agent which modify the binding of the other primary ligand indicate neutral cooperativity. The IC_{50}, or EC_{50}, of a test agent on the affinity ratio of either primary ligand corresponds approximately to the Kd of the test agent for the free receptor. High concentrations of compounds which show neutral or positive cooperativity with ^{3}H-NMS and which strongly inhibit ^{3}H-NMS dissociation may inhibit ^{3}H-NMS binding through a kinetic effect, i.e. lack of equilibration of ^{3}H-NMS binding.

Figure 10: Inhibition of ^{3}H-NMS binding to M_3 receptors by ACh, alone and in the presence of three concentrations of 2c (WIN 62577). GTP (0.2 mM) was present. The data were fitted to Equation 1 (see Methods) to yield a log affinity of 2c of 5.31, cooperativity with ^{3}H-NMS of 0.47, and cooperativity with ACh of 1.41. The inset shows affinity ratios (1/dose ratio) for ACh and ^{3}H-NMS, calculated from the parameters of the fit.

Figure 11: Dissociation of ^{3}H-NMS over time, alone and in the presence of three concentrations of 2a. For each receptor subtype, the parameter estimates and standard errors were derived from the nonlinear regression fits of the entire dataset to a version of Equations 2 and 3, where k_{off} and k_{offx} are the dissociation rate constants of ^{3}H-NMS from the free and 2a-liganded receptor respectively, and pK_{occ} is the log Kd of 2a for the ^{3}H-NMS-occupied receptor. The insets show the linearising transformation ln(B/Bo) vs. time, where B is the specific

binding remaining after a certain time and Bo is the initial level of specific binding.

Figure 12: Concentration-effect curves for 2a on ^{3}H-NMS dissociation, alone and in the presence of one or three concentrations of test agent, measured at a single time point. The data were converted to dissociation rate constants (see Methods) and expressed as a % of the control dissociation rate constant. The curves show the fits from nonlinear regression analysis to Equation 1. For strychnine and gallamine, dissociation of ^{3}H-NMS from the dually or triply liganded receptor was not different from 0 and the cooperative interaction with 2a was not different from 1 (i.e. neutral cooperativity), so these values were fixed. For 1a (KT 5720), 1f (staurosporine) and 2b (WIN 51708), the cooperative interaction with 2a was not different from 0 (i.e. competition), so this value was fixed. The panel marked 'dose-ratio plots' shows the log of the ratio $EC_{50\_+agent}/EC_{50\_alone}$ of 2a vs log[agent]. Each assay was repeated at least once, with similar results.

Detailed Description

Assays

As indicated above, the screening assays of the invention are useful in the identification of compounds capable of binding to the allosteric site disclosed herein and modulating the binding of a primary ligand to a muscarinic receptor. The precise format of these assays can be readily devised by the skilled person using the common general knowledge in the art. In one embodiment, the assays will employ (a) a muscarinic receptor of one of the M_1 to M_5 subtypes, (b) one or more primary ligands or ligand analogues, and (c) one or more candidate compounds. Optionally, the assays may additionally

employ (d) a compound known to act at the allosteric site which is capable of competing with the candidate compound being tested. The assays will generally involve contacting the receptor, the primary ligands or ligand analogues and one or more concentrations of the candidate compound *in vitro*, under conditions in which the candidate compound can bind or compete for binding at the allosteric site. The results of the assays can be determined by labelling one or more of the candidate compound, the competitive allosteric compound or the primary ligand or ligand analogue, and determining which species interact in the assay system.

In an alternative embodiment, and especially in the context of high throughput screening, it may be desirable that the screening assays involve determining whether the candidate compound binds to the allosteric site disclosed herein in the absence of the primary ligand. These assays could be followed with a separate determination of whether or in what sense the compounds binding to the allosteric site modulate the binding of a primary ligand or ligand analogue to the receptor. These assays could be carried out by contacting (a) a muscarinic receptor and (b) one or more candidate compounds, and optionally, (c) one or more compounds known to act allosterically, under conditions in which compounds (b) and/or (c) can bind or compete for binding to the allosteric site. The binding of the candidate compound or competitive compound to the receptor can be determined by labelling compounds (b) and/or (c).

The above assays may comprise carrying out controls, e.g. carrying out the assay in the absence or presence of the candidate compound(s).

In these assays, the muscarinic receptors can be present in either a free form or alternatively immobilised, e.g. on the surface of a cell expressing the receptor, or a solid support. A preferred format uses cell surface receptors.

The labelling of different types of agents is well known in the art. Broadly, this involves tagging the agent with a label or reporter molecule which can directly or indirectly generate detectable, and preferably measurable, signal. The linkage of reporter molecules may be direct or indirect, e.g. by a covalent bond or a non-covalent interaction. Examples of commonly used labels include fluorochrome, phosphor or laser dyes with spectrally isolated absorption or emission characteristics. Suitable fluorochromes include fluorescein, rhodamine, luciferin, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine. Other detectable labels include radioactive isotopic labels, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{126}I, or ^{99m}Tc, and enzyme labels such as alkaline phosphatase, β-galactosidase or horseradish peroxidase, which catalyze reactions leading to detectable reaction products and can provide amplification of signal.

Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes which catalyze reactions that develop or change colour or cause changes in electrical properties. They may be molecularly excitable, such that electronic transitions between

energy states result in characteristic spectral absorptions or emissions.

In the context of high throughput screening, the methods described herein can involve carrying out assays using groups or pools of candidate compounds, rather than individual compounds, to enhance the rate at which candidate compounds can be discarded. Individual groups of compounds having positive results in an assay can then be separated and screened to identify the compound(s) in the group which interact with the allosteric site and modulate the binding of the primary ligand. Appropriate measures should be taken to ensure that any one candidate compound is assayed with different pools of other candidate compounds. This protocol minimises the possible interfering masking effects of competitive antagonists or agonists which may be in one pool but not another.

The candidate compounds used may be natural or synthetic chemical compounds used in drug screening programmes. Mixtures of naturally occurring materials which contain several characterised or uncharacterised components may also be used.

Other candidate compounds may be based on modelling the 3-dimensional structure of a polypeptide or peptide fragment and using rational drug design to provide candidate compounds with particular molecular shape, size, hydrophobicity, hydrophilicity and charge characteristics.

The amount of candidate compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used.

Typically, from about 0.01 to 100,000nM concentrations of candidate compound may be used, for example 0.1 to 100μM.

In a further step, the method of the present invention may involve quantifying the amount of a candidate compound required to modulate the binding of the primary ligand to the muscarinic receptor by more detailed equilibrium or kinetic assays.

The screening methods of the invention may be followed by isolation and/or manufacture and/or use of a candidate compound selected in an assay, and/or further testing to determine whether a candidate compound having a positive, neutral or negative cooperative effect on the binding of the primary ligand to the muscarinic receptor has a biological property which makes it suitable for further development as a lead compound. These include tests to determine its activity in assays of function in membranes, whole cells, whole tissues and/or *in vivo*, as well as tests of its metabolic stability, bioavailability and duration of action and for the presence of side effects.

In a further aspect, the present invention provides the use of the above compounds in the design or screening for mimetics of the compounds which share the property of binding to the allosteric site of muscarinic receptors which binds to compound 1a and/or compound 2b.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a lead compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration. Mimetic design,

synthesis and testing may be used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. These parts or residues constituting the active region of the compound are known as its pharmacophore. Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are determined or modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be

screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for further testing or optimisation, e.g. *in vivo* or clinical testing.

Pharmaceutical Uses

The compounds identified herein as being useful for modulating the binding of a primary ligand to a muscarinic receptor can be formulated and used as pharmaceuticals.

The pharmaceutical compositions may comprise, in addition to one or more of the compounds, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, or intraperitoneal routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

The pharmaceutical formulations can be prepared by mixing the compounds of the present invention with one or more

adjuvants, such as excipients (e.g. organic excipients including sugar derivatives, such as lactose, sucrose, glucose, mannitol or sorbitol; starch derivatives, such as corn starch, dextrine or carboxymethyl starch; cellulose derivatives, such as crystalline cellulose, low hydroxypropyl-substituted cellulose, carboxymethyl cellulose, carboxymethyl cellulose calcium or internally bridged carboxymethyl cellulose sodium; gum arabic; dextran; and Pullulan; inorganic excipients including silicates, such as light silicic acid anhydride, synthetic aluminium silicate or magnesium meta-silicic acid aluminate; phosphates, such as calcium phosphate; carbonates, such as calcium carbonate; and sulphates, such as calcium sulphate); lubricants (e.g. metal stearates, such as stearic acid, calcium stearate or magnesium stearate; talc; colloidal silica; waxes, such as beeswax or spermaceti; boric acid; adipic acid; sulphates, such as sodium sulphate; glycol; fumaric acid; sodium benzoate; DL-leucine; sodium salts of aliphatic acids; lauryl sulphates, such as sodium laurylsulphate or magnesium laurylsulphate; silicates, such as silicic acid anhydride or silicic acid hydrate; and the foregoing starch derivatives); binders (e.g. polyvinyl pyrrolidone, Macrogol; and similar compounds to the excipients described above); disintegrating agents (e.g. similar compounds to the excipients described above; and chemically modified starch-celluloses, such as Crosscarmelose sodium, sodium carboxymethyl starch or bridged polyvinyl pyrrolidone); stabilisers (e.g. p-hydroxybenzoates, such as methylparaben or propylparaben; alcohols, such as chlorobutanol, benzyl alcohol or phenylethyl alcohol; benzalkonium chloride; phenols, such as phenol or cresol; thimerosal; dehydroacetic acid; and sorbic acid); corrigents (e.g. sweeteners, vinegar or perfumes, such as conventionally used); diluents and the

like.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as sodium chloride injection, Ringer's injection, lactated Ringer's injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Preferably, the pharmaceutically useful compound according to the present invention is given to an individual in a 'prophylactically effective amount' or a 'therapeutically effective amount' (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. Typically, this will be to cause a therapeutically useful effect in the patient, e.g. using the compounds to regulate the action of the primary ligand at a muscarinic receptor, and preferably one of the muscarinic receptor subtypes. The actual amount of the compounds administered, and rate and time-course of administration, will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's

Pharmaceutical Sciences, 16th edition, Oslo, A. (ed), 1980.

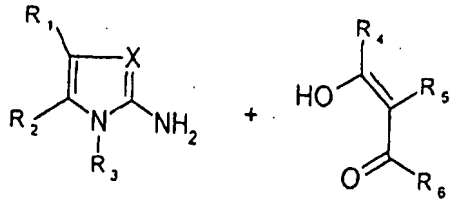
In particular, the compounds may be useful in the treatment of conditions mediated by the action of ACh at a muscarinic receptor. By way of example, these include Alzheimer's disease, Parkinson's disease, motion sickness, Huntingdon's chorea, schizophrenia, depression, anxiety, sedation, analgesia, stroke, preanaesthetic, antispasmodic, irritable bowel syndrome, bladder-incontinence or retention, peptic ulcer disease, bronchitis/asthma/chronic obstructive airway disease, sinus bradycardia, pacemaker regulation, glaucoma, achalasia, symptomatic diffuse oesophageal spasm, biliary dyskinesia, scleroderma, diabetes mellitus, lower oesophageal incompetence, intestinal pseudo obstruction, regulation of sleep, control of pupil diameter and non-ulcer dyspepsia.

Depending on the type and severity of condition, the composition can be administered to provide an initial dose of about 0.01 to 20 mg, more preferably 0.02 to 10 mg, of compound/kg of patient weight. As mentioned above, other dosing regimens and the determination of appropriate amount of the compounds for inclusion in the compositions can be readily determined by those skilled in the art.

Materials

^{3}H-NMS (81-86 Ci/mmol) was from Amersham International, UK, and ^{35}S-GTPγS (1000-1400 Ci/mmol) was from NEN, Boston. Brucine sulfate, gallamine triiodide and ACh chloride were from Sigma Chemical Co., Dorset, UK. Staurosporine was from Sigma and from Alexis Corporation, Nottingham, UK. Gö 7874 (1i), Gö 6976 (1h) and K-252c

(1g) were from Calbiochem, Nottingham, UK. K-252a (1b) and K-252b (1c) were from Alexis and from TCS Biologicals Ltd, Buckingham, UK. KT5823 (1e) and KT5720 (1a) were from TCS, Calbiochem and Alexis. KT5926 (1d) was from TCS and Calbiochem. WIN 51708 (2b) and WIN 62577 (2c) were from RBI (Semat), St Albans, UK. Analogues of 2 were synthesised using literature methods according to the general scheme:



with further chemical modification of the product as necessary. Further assistance for the synthesis of the compounds or related ones is provided in the papers by Bajwa and Sykes (1978-1980) cited in the references section.

Methods

Cell culture and membrane preparation:

CHO cells stably expressing cDNA encoding human muscarinic M_1-M_4 receptors (Buckley et al, 1989) were grown in alpha-MEM medium (GIBCO) containing 10% (v/v) new born calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM glutamine, at 37° under 5% CO_2. Cells were grown to confluence and harvested by scraping in a hypotonic medium (20 mM Hepes + 10 mM EDTA, pH 7.4). Membranes were prepared at 0°C by homogenization with a

Polytron followed by centrifugation (40,000 x g, 15 min), were washed once in 20 mM Hepes + 0.1 mM EDTA, pH 7.4, and were stored at -70°C in the same buffer at protein concentrations of 2-5 mg/ml. Protein concentrations were measured with the BioRad reagent using bovine serum albumin as the standard. The yields of receptor varied from batch to batch but were approximately 10, 1, 2 and 2 pmol/mg of total membrane protein for the M_1, M_2, M_3 and M_4 subtypes respectively.

Radioligand binding assays:

Unless otherwise stated, frozen membranes were thawed, resuspended in incubation buffer containing 20 mM Hepes + 100 mM NaCl + 10 mM $MgCl_2$ (pH 7.4) and incubated with radioligand and unlabelled drugs for two hours at 30°C in a volume of 1 ml. Membranes were collected by filtration over glass fibre filters (Whatman GF/B) presoaked in 0.1% polyethylenimine, using a Brandel cell harvester (Semat, Herts, UK), extracted overnight in scintillation fluid (ReadySafe, Beckman) and counted for radioactivity in Beckman LS6000 scintillation counters. Membrane protein concentrations (5-50 µg/ml) were adjusted so that not more than about 15% of added radioligand was bound. Nonspecific binding was measured in the presence of 10^{-6}M QNB (an antagonist with picomolar potency) and accounted for 1-5% of total binding. GTP was present at a concentration of $2x10^{-4}$M in assays containing unlabelled ACh. Data points were usually measured in duplicate. CHO cell membranes do not possess cholinesterase activity (Gnagey and Ellis, 1996; Lazareno and Birdsall, 1993) so ACh could be used in the absence of a cholinesterase inhibitor. The compounds were dissolved in dimethyl sulfoxide which, at the highest final concentration of 2%, had no effect on binding.

Experimental designs and data analysis:

General data preprocessing, as well as the 'affinity ratio' calculations and routine plots of the semiquantitative equilibrium assay, were performed using Minitab (Minitab Ltd, Coventry, UK). The other assays were analysed with nonlinear regression analysis using the fitting procedure in SigmaPlot (SPSS Inc., Erkrath, Germany). This procedure is relatively powerful in that it allows the use of two or more independent variables, e.g. concentrations of two drugs.

Equilibrium binding assays for estimation of the affinity of an allosteric agent for the receptor and the magnitude of its cooperativity with ^{3}H-NMS and ACh:

The design and analyses have been described in detail (Lazareno and Birdsall, 1995;Lazareno et al, 1998). Briefly, specific binding of a low concentration of ^{3}H-NMS (1-2 times the Kd) was measured in the presence of a number of concentrations of test agent, all in the absence and presence of one or more concentrations of ACh. Specific binding of a high concentration of ^{3}H-NMS (5-10 times Kd) was also measured. Nonlinear regression analysis was used to fit the data to the equation:

Equation 1

$$B_{LAX}=\frac{Bmax.L.K_L.(1+\alpha.(X.K_x)^s)}{1+(X.K_x)^s+(A.K_A)^n.(1+\beta.(X.K_x)^s)+L.K_L.(1+\alpha.(X.K_x)^s)}$$

where B_{LAX} is observed specific bound radioligand, L, A, and X are concentrations of ^{3}H-NMS, ACh and allosteric agent respectively, K_L, K_A and K_X are affinity constants for the corresponding ligands and the receptor, α and β are allosteric constants of X with ^{3}H-NMS and ACh

respectively, n is a logistic slope factor to describe the binding of ACh, and s is a 'Schild slope' factor to describe the binding of X. According to the allosteric model s should be 1.

Above a certain concentration, some allosteric agents, especially those which exhibit neutral or positive cooperativity with ^{3}H-NMS, may slow the kinetics of ^{3}H-NMS binding so much that the binding does not reach equilibrium. In most cases sufficient incubation time was used to allow ^{3}H-NMS binding in the presence of the agent to reach equilibrium. In a few cases, however, the highest concentration of agent would be predicted to slow ^{3}H-NMS kinetics sufficiently to prevent binding equilibrium from being reached, and in these cases the data were better fitted to the equation:

Equation 2

$$B_{LAXt}=B_{LAX}+(B_{L_o}-B_{LAX}).(\exp(\frac{-t.k_{off}}{1+\alpha.(X.K_X)^s}+\frac{t.k_{off}L.K_L}{1+(X.K_X)^s+(A.K_A)^n.(1+\beta.(X.K_X)^s)}))$$

where B_{LAXt} is observed specific binding under nonequilibrium conditions, B_{LAX} is the predicted equilibrium binding defined in Equation 1, t is the incubation time, k_{off} is the dissociation rate constant of ^{3}H-NMS, and B_{L0} is the initial amount of bound radioligand, set to zero in this case. This equation assumes that the dissociation of ^{3}H-NMS from the allosteric agent-occupied receptor is negligible, and that the binding kinetics of both ACh and the allosteric agent are fast in comparison with the dissociation rate of ^{3}H-NMS.

If only a single concentration of ACh was used, the data were visualised with 'affinity ratio' plots, where the affinity ratio is the apparent affinity of the 'primary' ligand (^{3}H-NMS or ACh) in the presence of a particular concentration of test agent divided by the apparent affinity of the primary ligand in the absence of test agent. Theoretically, the EC_{50} or IC_{50} of the affinity ratio plot corresponds to the Kd of the test agent at the free receptor, and the asymptotic level corresponds to the cooperativity constant for the test agent and primary ligand (Lazareno and Birdsall, 1995). Affinity ratios were calculated from the specific binding data as follows (Lazareno and Birdsall, 1999):

The affinity ratio of ^{3}H-NMS in the presence of a single concentration of test agent is given by the equation:

Equation 3

$$r_L = \frac{B_{LX}(B_{LI}-B_L)}{B_{LI}.B_L.(1-q)-B_{LX}(B_L-q.B_{LI})}$$

The affinity ratio of ACh the presence of a single concentration of test agent is given by the equation:

Equation 4

$$r_A = \frac{B_L.B_{LA}.(B_{LI}-B_L).(B_{LX}-B_{LAX})}{B_{LAX}(B_L-B_{LA}).[B_{LI}.B_L.(1-q)-B_{LX}(B_{LX}(B_L-q.B_{LI})]}$$

where B_L is binding in the presence of the low [^{3}H-NMS] alone; B_{LI} is binding in the presence of the high [^{3}H-NMS]; B_{LA} is binding in the presence of the low [^{3}H-NMS] and ACh; B_{LX} is binding in the presence of the low [^{3}H-NMS] and a particular concentration of test agent; B_{LAX} is

binding in the presence of the low [^{3}H-NMS], ACh and the same concentration of test agent; L is the low ^{3}H-NMS concentration; L_1 is the high ^{3}H-NMS concentration; and q is the ratio of low and high ^{3}H-NMS concentrations, L/L_1.

With assays containing a number of ACh concentrations, affinity ratio plots were calculated using the parameter estimates from the fit of the dataset to Equation 1 or 2 as appropriate (Lazareno and Birdsall, 1995).

The affinity ratio of ^{3}H-NMS and ACh, r_L and r_A respectively, are given by the equations:

Equation 5

$$r_L = \frac{1+\alpha.X.K_x}{1+X.K_x}$$

and equation 6

$$r_A = \frac{1+\beta.X.K_x}{1+X.K_x}$$

where the symbols are as described above.

Off-rate assay to estimate the affinity of an allosteric agent for the ^{3}H-NMS-occupied receptor:

A high concentration of membranes (2-4 mg protein/ml) was incubated with a high concentration of ^{3}H-NMS (5 nM) for about 15 minutes. Then 10 µl aliquots were distributed to tubes which were empty or contained 1 ml of 10^{-6}M QNB alone and in the presence of a number of concentrations of allosteric agent (typically n=4). Non-specific binding was measured in separately prepared tubes containing 10 µl membrane and 2 µl of ^{3}H-NMS + QNB. Some time later, about 2.5 dissociation half-lives (see Table 2), the samples were filtered. The data were transformed

to rate constants, k_{off}, using the formula:

$$k_{off}=\ln(B_0/B_t)/t$$

where B_0 is initially bound radioligand and B_t is bound radioligand remaining after t minutes dissociation. These values were finally expressed as % inhibition of the true ^{3}H-NMS dissociation rate constant (k_{off} in the absence of allosteric agent) and fitted to a logistic function using nonlinear regression analysis. Theoretically the curves should have slopes of 1, and correspond to the occupancy curves of the allosteric agents at the ^{3}H-NMS-occupied receptors, regardless of whether the inhibition of ^{3}H-NMS dissociation is caused by an allosteric change in the shape of the receptor or the trapping of the ^{3}H-NMS in its binding pocket by the bound allosteric agent (Lazareno and Birdsall, 1995). Initially the curve was fitted without constraints. If the slope factor was not different from 1, and the maximal inhibition ('Emax') did not exceed about 100%, then the slope was constrained to 1 and the Emax was fitted. If the fitted Emax exceeded 100% (a physical impossibility, apart from experimental variation or error) then the Emax was constrained to 100 and the slope fitted. With the compounds under study the Emax was often less than 100, and in most such cases the data were well fitted with the slope constrained to 1.

GTPγS binding assay:

Membranes expressing M_1 receptors (5-20 µg/ml) were incubated with ^{35}S-GTPγS (0.1 nM), GDP (10^{-7}M) and ligands in incubation buffer in a volume of 1 ml for 30-60 minutes at 30°C. Bound label was collected by filtration over glass fibre filters prewetted with water.

Part 1

Results

The structures of the compounds examined are shown on page 54. Figure 1 shows effects of compound 1f (staurosporine) on equilibrium ^{3}H-NMS binding at M_1 receptors in the absence and presence of a fixed concentration of ACh. ^{3}H-NMS binding was increased by staurosporine concentrations up to 10 μM and was reduced at 30 μM. The increase in ^{3}H-NMS binding reflects a decrease in the Kd of ^{3}H-NMS rather than an increase in Bmax (data not shown). The decrease in binding with 30 μM staurosporine is caused by the slowing of ^{3}H-NMS kinetics by high concentrations of staurosporine (see below) and the consequent lack of equilibration of ^{3}H-NMS binding (Lazareno and Birdsall, 1995). The effect of staurosporine on ACh binding is not clear from inspection of Figure 1, but nonlinear regression analysis of the data, which also takes into account the effects of high concentrations of staurosporine on the kinetics of ^{3}H-NMS, provided a good fit to the data (lines in Figure 1) and revealed a 4-fold negative cooperativity between ACh and staurosporine. The independent effects of staurosporine on ^{3}H-NMS and ACh binding across the four receptor subtypes are easier to visualise when the binding data are transformed into affinity ratios (Lazareno and Birdsall, 1995;Lazareno et al, 1998) (Figure 2). In theory, the EC_{50} or IC_{50} of the affinity ratio plot corresponds to the Kd of the test agent for the free receptor, and the asymptotic value corresponds to the cooperativity with the primary ligand. Staurosporine (1f) showed positive cooperativity with ^{3}H-NMS at M_1 and M_2 receptors, neutral cooperativity with ^{3}H-NMS at M_4 receptors and was inactive or neutrally cooperative at M_3 receptors. It had negative cooperativity with ACh at M_1, M_2 and M_4 subtypes and was neutral with ACh or inactive at

M_3 receptors. Staurosporine had Kd values for unoccupied receptors in the μM range (Figure 2, Table 1). In two functional assays with M_1 receptors measuring the stimulation by ACh of ^{35}S-GTPγS binding, 10 μM staurosporine reduced basal activity and the Emax by 17% ± 7% and 25% ± 4% respectively, and also caused a 2.9 ± 0.9 fold decrease in the potency of ACh, which is consistent with the 3.6-fold change predicted from the ^{3}H-NMS binding studies (data not shown).

Staurosporine also inhibited ^{3}H-NMS dissociation (Figure 3). All the curves had slope factors of 1. Staurosporine was most potent and effective at M_1 receptors, causing apparently complete inhibition of ^{3}H-NMS dissociation with an IC_{50} of 1 μM (Table 2). It was 3-4-fold weaker at the other receptor subtypes, and also caused submaximal inhibition of ^{3}H-NMS dissociation, with the smallest effect, 67% inhibition, seen at M_3 receptors. The IC_{50} values for the inhibition of ^{3}H-NMS dissociation correspond in theory to the Kd values of staurosporine for the ^{3}H-NMS-liganded receptors, and the values at M_1 and M_2 receptors are consistent with the values predicted from the equilibrium binding studies according to the allosteric model (Table 2). There was a 2-fold disparity between predicted and observed values at M_4 receptors, probably because of inaccuracies in measuring the small degree of negative cooperativity with ^{3}H-NMS. In equilibrium binding studies at M_3 receptors staurosporine had little or no effect on the binding of either ^{3}H-NMS or ACh: the clear inhibition of ^{3}H-NMS dissociation caused by staurosporine over the same concentration range suggests that staurosporine was neutrally cooperative with ^{3}H-NMS and ACh at M_3 receptors, rather than inactive.

Gö 7874 (1i), a ring-opened analogue of staurosporine still bearing a positive charge, showed weak negative cooperativity with ^{3}H-NMS and stronger negative cooperativity with ACh at M_1, M_2 and M_4 receptors, and the reversed pattern at M_3 receptors (Figure 2). It was necessary to introduce a slope factor >1 into the binding equation for Gö 7874 in order to fit the data adequately to the allosteric model (Table 1). Gö 7874 caused apparently complete inhibition of ^{3}H-NMS dissociation at M_1, M_2 and M_4 receptors, and submaximal inhibition at M_3 receptors. The slopes of the curves at M_1, M_2 and M_4 receptors were also >1 (Figure 3, Table 2). The ternary complex allosteric model does not predict slope factors different from 1, so it cannot provide a complete mechanistic explanation of the data. Nevertheless, the affinity values of Gö 7874 for the ^{3}H-NMS-occupied receptor predicted by the model from the equilibrium binding studies are in excellent agreement with the observed values at M_1 and M_4 receptors (Table 2), and show only a 3-fold discrepancy at M_2 and M_3 receptors, caused possibly by a combination of inaccuracies in the measurement of the small cooperative effects which occurred in equilibrium studies at the M_2 and M_3 subtypes (Table 1) and the small inhibitory effect on ^{3}H-NMS dissociation from M_3 receptors.

KT5823 (1e), a ring-contracted analogue of staurosporine in which the methylamino group is replaced by a methyl ester, caused a large increase in ^{3}H-NMS binding at M_1 and M_2 receptors, and showed neutral or small positive cooperativity with ACh at these receptors. KT5823 was inactive or neutrally cooperative with ^{3}H-NMS and ACh at M_3 and M_4 receptors (Figure 2). The positive cooperativity with NMS at M_1 receptors was confirmed in functional studies in which 1mM KT5823 increased the

potency of ACh 1.9 ± 0.9 fold at M_1 receptors for stimulating ^{35}S-GTPγS binding, and caused a 3.3 ± 1.7 fold increase in the affinity of unlabelled NMS (n=2, data not shown). KT5823 inhibited ^{3}H-NMS dissociation completely at M_1 receptors, 80% at M_2 receptors and 30-40% at M_3 and M_4 receptors (Figure 3). The affinity of KT5823 for the ^{3}H-NMS-occupied receptor estimated from equilibrium studies at M_1 and M_2 receptors was very similar to the values measured directly. The inhibition of ^{3}H-NMS dissociation seen at M_3 and M_4 receptors may indicate that KT5823 is neutrally cooperative with ^{3}H-NMS and ACh at these receptors, rather than inactive.

KT5720 (1a), a hexyl ester analogue of KT5823, was positively cooperative with both ^{3}H-NMS and ACh at M_1 receptors (Figure 1, Table 1). The small (40%) increase in ACh affinity was confirmed in more detailed assays (Figure 5). KT5720 had little or no effect at M_3 receptors, and showed neutral cooperativity with ^{3}H-NMS and negative cooperativity with ACh at M_4 receptors. The effects of KT5720 at M_2 receptors are unclear: earlier batches had small inhibitory effects with ^{3}H-NMS and ACh (Figure 2), while a later batch had small positive effects with ^{3}H-NMS (data not shown): no batch-dependent effects were noted at the other subtypes. KT5720 caused incomplete inhibition of ^{3}H-NMS dissociation at M_1, M_3 and M_4 receptors, with little or no effect at M_2 receptors (Figure 3). The largest effect was seen with M_1 receptors, and, at this subtype alone, low concentrations of KT5720 caused a small but consistent increase in ^{3}H-NMS dissociation. This phenomenon was observed in 10 out of 11 single-time point assays, with the dissociation rate constant (k_{off}) of ^{3}H-NMS increased by 11 ± 1 % (n=10) in the presence of the most effective concentration between 10 and 300 nM KT5720, and in two full time course studies

in which k_{off} in the presence of 0.1 μM KT5720 was increased by 16.3 ± 0.5 % (data not shown). The affinity of KT5720 for the ^{3}H-NMS-occupied receptor estimated from equilibrium studies at M_1 and M_4 receptors was similar to the values measured directly (Table 2).

K-252a (1b), in which the methoxy group of KT5823 is replaced by a hydroxyl group, showed positive cooperativity with ^{3}H-NMS at M_1 receptors and neutral or small negative cooperativity with ACh (Figure 2, Table 1). Little or no effect was seen in equilibrium binding studies with the other subtypes. K-252a inhibited ^{3}H-NMS dissociation at M_1 receptors, apparently by 100%. Slope factors > 1 were required to fit the data adequately. Only small, though consistent, effects on ^{3}H-NMS off rate were seen at the other subtypes (Figure 3, Table 2).

K-252b (1c), K-252c (1g), KT-5926 (1d) and Gö 6976 (1h) at concentrations up to 10 μM had little or no effect on equilibrium binding of ^{3}H-NMS and ACh and on ^{3}H-NMS dissociation (data not shown) and were not studied further.

We have attempted to determine whether some of the allosteric effects described above occurred through an interaction at the same site on the receptor at which other known allosteric agents act. Figure 5a shows the interaction between KT5720 (1a) and gallamine on equilibrium ^{3}H-NMS binding at M_1 receptors. Gallamine had its expected inhibitory effect on ^{3}H-NMS binding, and KT5720 showed the expected positive cooperativity with ^{3}H-NMS. If gallamine and KT5720 were acting at the same site then gallamine should have become less potent in the presence of KT5720 and the nonlinear regression analysis would have indicated strong negative cooperativity

between the two agents. In fact, the analysis revealed neutral cooperativity, i.e. in equilibrium binding studies gallamine and KT5720 interact allosterically at M_1 receptors through distinct and apparently non-interacting sites. In similar experiments with staurosporine and gallamine at M_1 receptors, however, there was an negatively cooperative or competitive interaction between the compounds (Figure 5b).

In order to study the site(s) on the M_1 receptor at which KT5720 acts to affect ^{3}H-NMS dissociation, the concentration-related effect of KT5720 was measured alone and in the presence of two or three concentrations each of gallamine, brucine and staurosporine. Very similar results were obtained in two independent assays, and the combined data are shown in Figure 6. The data in each condition are shown in two forms: as percentage inhibition of the overall control (i.e. 'true') k_{off} measured in the absence of any test agent, and, for each curve, as a fraction of its own control k_{off} measured in the presence of test agent and the absence of KT5720. This latter 'fractional effect' measure has useful properties: if the interaction between KT5720 and the test agent is competitive, then in the presence of test agent the EC_{50} will increase and the asymptotic 'fractional effect' will also change; if the interaction is noncompetitive and noninteracting (i.e. with neutral cooperativity), and if maximal concentrations of test agent completely inhibit ^{3}H-NMS dissociation, then in the presence of test agent both the EC_{50} and asymptotic levels are unchanged.

The lines in the top panel of Figure 6 (except in the presence of staurosporine) are hyperbolic fits to the data. The effect of low concentrations of KT5720 to

increase ^{3}H-NMS dissociation was apparent in all the curves. When the data are expressed as a fractional effect of own control, the curves for KT5720 in the presence of various concentrations of gallamine or brucine overlap, i.e. they have the same EC_{50} and asymptotic level. There was a small concentration-related increase in potency in the presence of gallamine, but this is probably experimental noise, since a positively cooperative interaction would result in decreases in the asymptotic level of the 'fraction of own control' plots. These data therefore demonstrate that KT5720 acts at a different site from the site(s) at which gallamine and brucine act to inhibit ^{3}H-NMS dissociation from M_1 receptors.

A quite different pattern of results was seen with staurosporine. The stimulating effect of low concentrations of KT5720 became more apparent, and the curves tend to converge at high concentrations of KT5720 more than in the presence of gallamine or brucine. It was not possible to measure EC_{50} values accurately, but inspection of the 'fractional effect' plot suggests that staurosporine reduced the potency of KT5720. These results indicate that staurosporine and KT5720 compete for the site which mediates inhibition of ^{3}H-NMS dissociation. They strongly suggest that staurosporine can act at different site(s) from gallamine or brucine.

Discussion

Five of the nine indolocarbazoles which we have studied act allosterically at muscarinic receptors. Of these, four have similar structures and a number of similarities in their allosteric effects, while the fifth, Gö 7874 (1i), lacks the tetrahydrofuran/pyran ring system, which may account for its somewhat different effects.

In equilibrium binding studies, the four active staurosporine-like compounds (staurosporine (1f), KT5823 (1e), KT5720 (1a) and K-252a (1b)) showed only positive or neutral cooperativity with ^{3}H-NMS, or were apparently inactive, while positive, neutral and negative cooperativity was observed with ACh. The four compounds showed their highest affinity, and largest positive effects with ^{3}H-NMS, at the M_1 receptor, while they were inactive (or neutrally cooperative with ^{3}H-NMS and ACh) at M_3 receptors. These compounds bound with slope factors of 1, except for KT5823 at M_1 receptors, and this exception may be partly accounted for by artefacts arising from the strong (7-10 fold) positive cooperativity with ^{3}H-NMS seen with this compound. Gö 7874 (1i), the other positively charged ligand in addition to staurosporine, also showed selectivity for the M_1 receptor but, in contrast to the other four compounds, it showed negative cooperativity with ^{3}H-NMS, and both neutral and negative cooperativity with ACh, and it bound with slope factors greater than 1.

The four staurosporine-like compounds also showed selectivity for the ^{3}H-NMS-occupied M_1 receptor, but this was manifest more clearly in the magnitude of inhibition of ^{3}H-NMS dissociation than in the affinity. Again, these compounds bound to the ^{3}H-NMS-occupied receptor with slopes of 1, except for K-252a at M_1 receptors. Gö 7874 inhibited ^{3}H-NMS dissociation completely from M_1, M_2 and M_4 receptors with slope factors significantly greater than 1.

There seems to be a relationship between the activity of the compounds in equilibrium binding assays and the maximum degree of inhibition of ^{3}H-NMS dissociation: an ad hoc correlation for the current data is that compounds showing less than 50% inhibition of ^{3}H-NMS dissociation at

a particular subtype appear inactive in equilibrium studies, while those slowing ^{3}H-NMS dissociation by > 50% show activity in equilibrium studies. This rule works in 17/20 cases, the exceptions being staurosporine at M_3, Gö 7874 at M_3 and KT5720 at M_4 receptors. The positive relationship between allosteric activity at equilibrium and the degree of inhibition of ^{3}H-NMS dissociation may reflect the degree to which binding of the allosteric agent perturbs the primary ligand recognition site on the receptor. Those cases where the test agent inhibits ^{3}H-NMS dissociation but appears to be inactive at equilibrium may actually reflect a lack of cooperative effect, i.e. neutral cooperativity, rather than a lack of binding of the test agent at equilibrium.

According to the allosteric model, the affinity of a test agent for the ^{3}H-NMS-occupied receptor may be estimated in two independent ways: from direct measurement of effects on ^{3}H-NMS dissociation, and from the product of affinity for the free receptor and cooperativity with ^{3}H-NMS, measured at equilibrium. In this study there are 11 instances where these measures have been determined with sufficient precision to allow comparison. There was good agreement between the measures: three comparisons differed by about 3-fold, one by about 2-fold, and the rest (7) by 60% or less, and there was no obvious bias since in 5 cases the equilibrium estimate was larger than the directly measured value and in 7 cases it was smaller. These results suggest that the data can be accounted for by the allosteric model, even though the steep slopes seen with Gö 7874 (1i) and K-252a (1b) are not predicted by the model.

The simple model also cannot account for the effects of KT5720 on ^{3}H-NMS dissociation at M_1 receptors, with an

initial speeding of dissociation by about 15 % at submicromolar concentrations, followed by submaximal inhibition of dissociation at higher concentrations. In the presence of staurosporine the speeding effect became more prominent, while the potency of KT5720 for slowing ^{3}H-NMS dissociation appeared to be reduced, suggesting that KT5720 may be exerting its effects at two distinct sites, only one of which can also be occupied by staurosporine. In contrast, the presence of gallamine or brucine had no effect on the potency of KT5270 or its fractional asymptotic effect, suggesting that, unlike staurosporine, gallamine and brucine act at a different site from the site(s) by which KT5720 modulates ^{3}H-NMS dissociation, and that there is no interaction (i.e. neutral cooperativity) between the binding of KT5270 and that of brucine or gallamine.

A similar conclusion can be drawn from equilibrium binding studies at M_1 receptors, in which KT5720 showed no interaction with gallamine. In contrast, similar equilibrium binding studies at M_1 receptors with staurosporine and gallamine revealed a negatively cooperative or competitive interaction. The different interactions with gallamine shown by staurosporine (negative) and KT5720 (neutral) may be related to the fact that staurosporine, like gallamine, is a positively charged molecule, whereas KT5720 is neutral.

These results demonstrate that KT5720, and possibly other indolocarbazoles, bind to an allosteric site on muscarinic receptors which is distinct from the 'common allosteric site' to which gallamine and most other allosteric agents bind. Previously reported allosteric agents have a positively charged nitrogen which is thought to be important for their action. Staurosporine

and Gö 7874 are also positively charged, but the other active indolocarbazoles are neutral, which suggests that there is no necessity for a positively charged nitrogen at this new allosteric site. The observed affinities and cooperativities are sensitive to small changes in the chemical structure of the analogues. For example, increasing the alkyl chain length of the ester function of K-252a or methylation of its hydroxyl group increase affinity 3-15-fold, whereas removal of the methyl group on the ester of K-252a or the alkoxy substitution of the indolocarbazole ring generate apparently inactive compounds.

The agents studied here are known to be potent inhibitors of various protein kinases, and in most cases the agents have much higher affinity for these targets than for muscarinic receptors, but it is worth noting that KT5720 has only about 6-fold higher potency for its preferred target, protein kinase A (PKA), than for the M_1 receptor (log affinity of 7.2 at PKA vs. 6.4 at M_1 receptors).

One of our aims has been the development of drugs which enhance the affinity of ACh at M_1 receptors while having no effect on ACh binding and function at the other subtypes. The detection of the allosteric properties of KT5720 may be a step towards that goal. KT5720 was the most potent compound at M_1 receptors with a log affinity for the free receptor of 6.4, and it showed a small (40%) but consistent positive cooperative effect with ACh. In addition it had little or no effect on ACh affinity at the other subtypes, so KT5720 is close to displaying an 'absolute subtype selectivity' for the M_1 receptor, i.e. a positive or negative interaction with ACh at one receptor subtype and neutral cooperativity at the others, so that whatever concentration of agent is administered only the

one receptor subtype is affected functionally (Lazareno and Birdsall, 1995).

The results in this section show a quite potent allosteric interactions of staurosporine and some other indolocarbazole analogues at muscarinic receptors which, at least in the case of KT5720, occur at a site distinct from the 'common allosteric site'. The active indolocarbazoles cause different maximal effects on ^{3}H-NMS dissociation, and the size of the maximal effect on ^{3}H-NMS dissociation is a good predictor of the activity detected in equilibrium studies, suggesting a common mechanism for the two effects. In general the results from equilibrium and dissociation assays were mutually consistent with the ternary allosteric complex model as the underlying mechanism of the observed effects. Finally, KT5720 is the most potent agent described so far showing positive cooperativity with ACh at M_1 receptors.

Part 2

Results

We also examined the interaction of a second family of compounds represented by general formula 2 shown on pages 55 and 56. Figure 7 shows representative data from off rate assays for these structures, and the parameter estimates are summarised in Table 3. Figures 8 and 9 show representative affinity ratio plots from equilibrium assays, and the parameter estimates are summarised in Table 4.

2b (WIN 51708) strongly inhibited ^{3}H-NMS dissociation at M_2 and M_4 receptors, with about 10-fold M_4 selectivity, it caused submaximal inhibition at the M_1 receptor and had no effect at the M_3 receptor. 2c (WIN 62577), containing a 5-6 double bond, which reduced the affinity for the

^{3}H-NMS-occupied M_4 receptor by about 10-fold and led to a smaller maximal effect at M_2 receptors. These effects were reflected in the equilibrium assays, where 2b showed a small degree of positive cooperativity with ^{3}H-NMS at M_2 receptors and a larger positive effect at M_4 receptors, while 2c, which was up to 5-fold less potent, showed only small negative cooperativity with ^{3}H-NMS. With respect to ACh, 2b showed small negative cooperativity at M_1 and M_3 receptors and larger negative effects at M_2 and M_4 receptors, while 2c was also negative at M_2 and M_4 receptors, was almost neutral at M_1 receptors, and showed a small (1.5-fold) positive interaction with ACh at M_3 receptors. This latter effect was confirmed in more detailed assays (e.g. Figure 10). It is worth noting that the potency and small degree of negative cooperativity with ^{3}H-NMS of both 2b and 2c at M_3 receptors should result in some activity at M_3 receptors in the off rate assay, whereas no activity was observed.

Removal of the bridgehead nitrogen from 2c gave 2a and led to a 10-fold increase in affinity at M_1 and M_2 receptors, a 30-fold increase at M_3 receptors, but little or no change in affinity at M_4 receptors. With a log affinity of 6.5 it was the most potent of these compounds at M_1 receptors. It showed 2-5 fold negative cooperativity with both ^{3}H-NMS and ACh across the receptor subtypes. In the off rate assay 2a had the unique effect of speeding ^{3}H-NMS dissociation. It caused a 2-3 fold increase in ^{3}H-NMS off rate at M_3 receptors, with smaller effects at the other subtypes, with the order of effectiveness $M_3>M_1>M_4>M_2$. This effect was confirmed in full dissociation assays (Figure 11).

Replacement of the ethynyl and hydroxy groups of 2a with a keto function, in 2f and 2i, resulted in a complete

loss of activity in the off rate assay (Figure 7) and the equilibrium assay (data not shown).

Removal of the ethynyl substituent from 2b and 2c gave rise to the 17-hydroxy analogues, 2d and 2e. The compounds had slightly greater potency than their corresponding analogues in the off rate assay, and larger inhibitory effects at M_1 receptors, but still little or no effect at the ^{3}H-NMS-occupied M_3 receptor. In equilibrium studies (Figure 8) 2d showed positive cooperativity with ^{3}H-NMS at all subtypes, and its log affinity of 7 at the free M_4 receptor was the most potent interaction in this study. 2e showed small negative, neutral and positive effects with ^{3}H-NMS. Both compounds had negative cooperativity with ACh, except 2e which showed a small (30%) positive cooperativity with ACh at M_3 receptors.

2g and 2j are analogues of 2b lacking two rings of the steroid moiety of 2b. 2g is reported to be the trans isomer and 2j is reported to be the cis isomer. 2b itself has the trans configuration, so it is very surprising that 2g was virtually inactive in the off rate assay (Figure 7) and equilibrium assays (data not shown), while 2j showed strong activity in the off rate assay (albeit 10-100 fold weaker than 2b), and in equilibrium assays, where it was strongly negative with ^{3}H-NMS and ACh, and only 2-5 fold less potent than 2b.

2l is a truncated form of 2j, and no longer chiral. It was also active, showing similar potency to 2j in the off rate assay (and a bigger effect on M_3 receptors), but less potency in equilibrium assays, especially at M_2 and M_4 receptors (Figure 9), leading to positive cooperativity with ^{3}H-NMS at these subtypes.

2h is the pyrimidoimidazole analogue of 2c, lacking the fused benzene ring. This change caused a reduction in affinity for both free the ^{3}H-NMS-occupied receptor of 2-20 fold, and stronger negative cooperativity with ^{3}H-NMS and ACh.

2k is an analogue of 2h with the imidazole ring attached to a different portion of the pyrimidine ring. It had a 30-200 fold higher affinity than 2h at ^{3}H-NMS-occupied receptors, or more than 1000-fold at M_3 receptors if the small effects at this subtype have been correctly interpreted. It also had 10-50 fold higher affinity for the free receptor than 2h, with Kd values of less than 1 mM at all subtypes. It showed strong positive cooperativity with ^{3}H-NMS at M_1 receptors (Figure 9), and was positive with ^{3}H-NMS at M_2 and M_4 receptors and weakly negative at M_3 receptors. It had 2-4 fold negative cooperativity with ACh.

2t, an analogue of 2k but with a pyrimidoimidazole substituent on the 16,17 positions of the steroid backbone, was inactive, reinforcing the importance of substitution in this region for activity.

Most compounds which bind to the primary or allosteric sites of muscarinic receptors have a basic nitrogen, though neutrally charged antagonists have been described. The two steroid structures (2m and 2n), which do not contain nitrogen, correspond to the steroid portion of 2c, 2h, 2a and 2k. Surprisingly, they show activity in both the off rate and equilibrium assays, and 2m seems to be more potent than 2h in the off rate assay (Figure 7). The 17-keto analogue, 2s, and the 2-substituted analogues, 2o-2r appeared to be inactive.

With regard to slope factors, most of the compounds had binding slopes of 1 in equilibrium or kinetic assays. The relatively weak compounds 2l, 2j and 2h however had steeper slopes indicating a more complex interaction.

The effect of 2a to increase ^{3}H-NMS dissociation provides an opportunity to assess whether 2a binds to the same site on the ^{3}H-NMS-occupied receptor as other allosteric agents. The dissociation rate constant (k_{off}) of ^{3}H-NMS from the M_3 receptor was measured at a single time point alone and in the presence of a range of concentrations of 2a, alone and in the presence of one or more concentrations of a second agent. The data were expressed as % of control k_{off}, as described in Methods, and fitted to Equation 1. The results are shown in Figure 12 and Table 6. Strychnine and gallamine reduced the Emax of 2a, indicating that they were acting at a different site from that occupied by 2a, but they did not affect the EC_{50} of 2a, indicating that there was no cooperative interaction, i.e. they showed neutral cooperativity. In contrast, 1a (KT 5720), 1f (staurosporine) and 2b (WIN 51708) did not appear to alter the E_{max} of 2a but reduced its potency, and the data were well fitted assuming a competitive interaction, although a strongly negative interaction cannot be ruled out. These results demonstrate that there are two allosteric sites on the M_1 and M_3 receptors. It is worth noting that 2b (WIN 51708) clearly binds to the ^{3}H-NMS-occupied M_3 receptor, even though it does not modify the k_{off} of ^{3}H-NMS.

Discussion

This paper describes a new series of compounds which interact allosterically with muscarinic receptors. The initial lead was provided by two commercially available

compounds, 2b (WIN 51708) and 2c (WIN 62577). These compounds are potent antagonists at rat NK_1 receptors, but the affinity of these compounds, exemplified by 2b, is reduced 400 fold at the human NK_1 receptor. In fact our results show that 2b is up to 60 fold more potent on human muscarinic receptors compared to human neurokinin receptors.

These two compounds and the analogues described in this paper exhibit positive, neutral and low negative cooperativities with NMS and especially ACh. This latter characteristic is important in that it suggests that the allosteric enhancers at a specific muscarinic receptor subtype may be synthesised in this series which are neutrally cooperative with ACh (and therefore inactive at any concentrations) at other subtypes. This form of selectivity, based on cooperativity rather than affinity, has been termed 'absolute subtype selectivity' (Lazareno et al, 1998; Birdsall et al, 1999) and is a direct consequence of the ternary complex allosteric model (Lazareno and Birdsall, 1995) which underpins the analyses of all our binding and functional data.

The ternary complex allosteric model implies that the affinity of a compound for the ^{3}H-NMS-occupied receptor can be estimated in two ways: as the product of affinity for the free receptor and cooperativity with 3H-NMS from equilibrium assays, and as the reciprocal of the IC50 (or EC50) from off rate assays. Table 5 shows a comparison between the pKocc (from equilibrium studies) and the pKoff (from off rate studies) values of those compounds for which there are at least two observations of each type of measure. Of the 32 comparisons, 23 show a discrepancy between the two measures of less than 0.3 log units (2-fold), a value which is readily accounted for by

experimental error. In most other cases the discrepancy can be accounted for by inaccuracies either observed in the data or predicted from the effect of the compound. The discrepancies of 2b and 2c at M_1 receptors may be explained by the small effects in the off rate assay, 2d has small effects at the M_3 receptor in the off rate assay, and at the M_4 receptor lower concentrations should have been used to define better the parameters. 2j has strong negative cooperativity at M_2 and M_4 receptors which cannot be measured accurately with the experimental designs used here, and 2k has very small effects in the off rate assay with M_3 receptors. That leaves the 3-fold discrepancies for both 2d and 2k at M_2 receptors, for which there are no obvious explanations. Two outliers out of 32 observations may be within the expected variability of such data, and overall the data pass this rather stringent test and are therefore consistent with the ternary complex allosteric model as the underlying mechanism which is responsible for effects on both equilibrium binding and on ^{3}H-NMS dissociation.

2b, 2c and and the analogues examined can be considered in simplistic terms as a fusion of a planar aromatic heterocyclic system with an alicyclic ring system, especially a steroid structure. Most surprisingly both the steroid moiety alone, for example 2n, (but not some other analogues) and the heterocyclic ring system (21) are individually capable of interacting allosterically with ^{3}H-NMS and with comparable affinities to each other. This result implies that these compounds may interact with different but contiguous subdomains of the same pharmacophore.

Binding to the allosteric site is sensitive to the nature of the heterocycle when the steroid ring is kept constant

(compare 2h, 2a and 2k with 2c). Equally the nature of the 17-substituent on the steroid ring seems important in the fused systems. The analogues with a 17-keto function appear inactive, whereas all compounds with a 17β hydroxyl group are active. The presence of the 17-α ethynyl group has more subtle effects (mainly on the M_4 receptor) and these seem to be interrelated with effects of the saturation status of the 5-6 bond.

Another surprising result is that 2j is active whereas 2g appears to be inactive. Both these compounds are racemates and they represent truncated analogues of 2b with the AB rings of the steroid in the cis and trans configurations respectively. Activity was expected to be associated with the trans isomer rather than the cis isomer.

Many of the compounds investigated in this study, in contrast to most muscarinic allosteric agents, do not inhibit the association and dissociation of ^{3}H-NMS completely at high concentrations. They often only produce a 2-fold or less slowing effects on the kinetics and in some instances, especially at M_3 receptors, very small effects indeed (Figure 7).

The remarkable finding is that 2a increases the dissociation rate of ^{3}H-NMS with the largest effect being observed at M_3 receptors and the smallest effect at M_2 receptors. It is noteworthy that the data from equilibrium and off rate studies with 2a are entirely consistent with the allosteric model, i.e. there are no discrepancies between estimates of affinity for the ^{3}H-NMS-occupied receptor from the two types of assay, and the slope factors were 1 or close to 1. The agreement occurs despite the enhancement of ^{3}H-NMS dissociation by

2a being the opposite to the inhibitory effect seen with all the other compounds in this study, and in every other published study of allosteric agents at muscarinic receptors.

This unique effect of 2a allowed us to assess whether other allosteric agents inhibit ^{3}H-NMS dissociation by acting at the same site as 2a. We found that 1a (KT5720), 1f (staurosporine) and 2b bind to the same site as 2a on the ^{3}H-NMS-liganded receptor, but gallamine and strychnine bind to a different site and have no effect on the binding of 2a, i.e. gallamine and strychnine show neutral cooperativity with 2a.

In conclusion, the following general points can be made. (1) allosteric agents can enhance, inhibit, or have no effect on the dissociation rate of ^{3}H-NMS. (2) There are at least two nonoverlapping allosteric sites, the 'common' site and the 'WIN' site; (3) Both allosteric sites can support a positive cooperative interaction with ACh; (4) The use of 2a provides a test of whether another allosteric agent binds to the 'WIN' site.

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The references mentioned herein are all expressly incorporated by reference.

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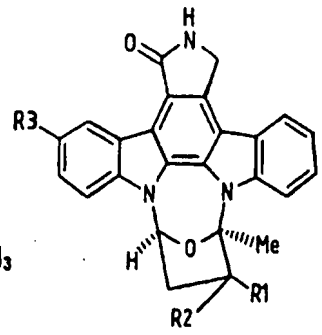
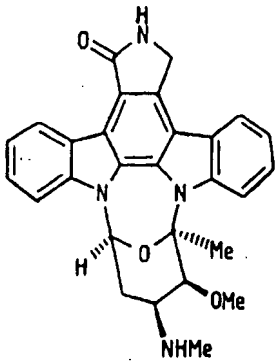
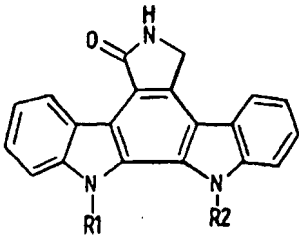
		R1	R2	R3
1a	KT5720	OH	$COO(CH_2)_5CH_3$	H
1b	K-252a	OH	$COOCH_3$	H
1c	K-252b	OH	COOH	H
1d	KT5926	OH	$COOCH_3$	$O(CH_2)_2CH_3$
1e	KT5823	OCH_3	$COOCH_3$	H



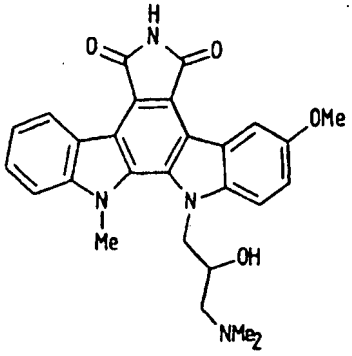
1f Staurosporine



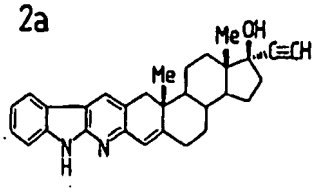
		R1	R2
1g	K-252c	H	H
1h	Gö 6976	CH_3	CH_2CH_2CN



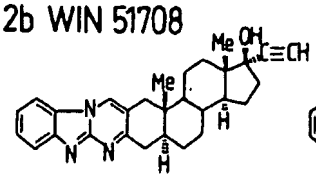
1i Gö 7874 HCl



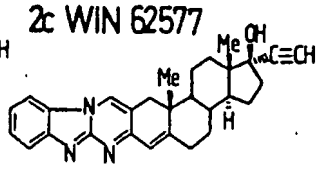
2a



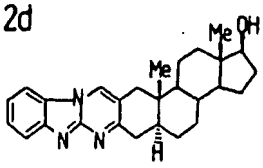
2b WIN 51708



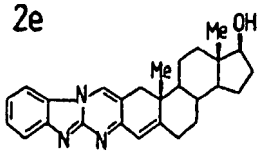
2c WIN 62577



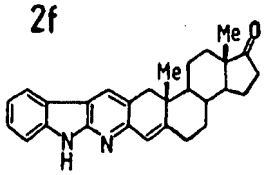
2d



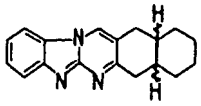
2e



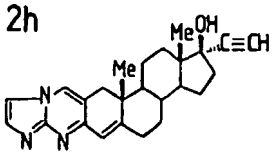
2f



2g trans



2h



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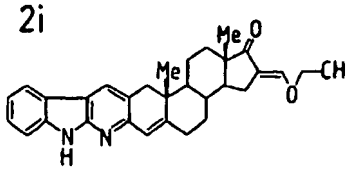

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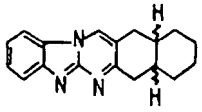

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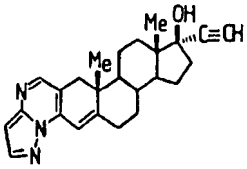

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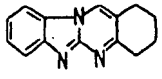

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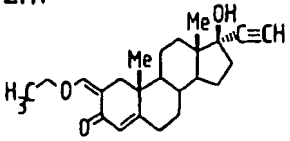

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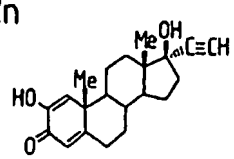

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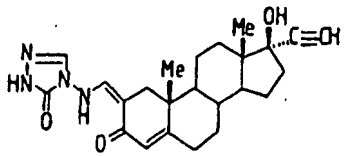

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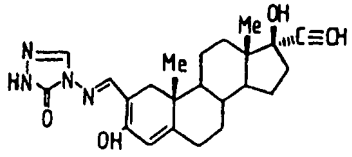

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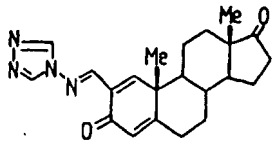

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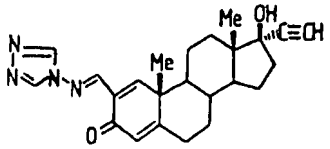

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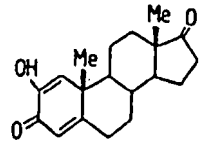

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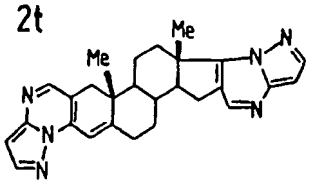

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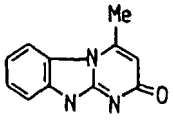


Table 1

Equilibrium binding parameters of indolocarbazoles with ^{3}H-NMS and Ach at muscarinic receptors. Assays such as those shown in Figures 2 and 5 were fitted to Equation 1 or 2 as appropriate (see Methods). The results are from at least 3 assays, except * n=2. Empty cells indicate that it was not possible to obtain at least 2 sets of parameter estimates.
#Some, but not all, of the values were obtained from analyses in which the affinity for the ^{3}H-NMS occupied receptor was fixed at the mean value obtained from offrate assays and shown in Table 2.

			M_1				M_2			
					cooperativity				cooperativity	
name			pK	slope	^{3}H-NMS	ACh	pK	slope	^{3}H-NMS	ACh
1a	KT 5720	mean	6.42#	1.00	1.94	1.39	5.29	1.05	1.56	0.82
		sem	0.09	0.00	0.14	0.09	0.33	0.05	0.60	0.45
1b	K-252a	mean	5.13*#	1.00	2.62	0.86				
		sem	0.14	0.00	0.68	0.13				
1e	KT 5823	mean	5.70	1.44	8.11	1.31	5.67*	1.00	3.27	1.34
		sem	0.05	0.14	1.61	0.44	0.15	0.00	0.19	0.03
1f	staurosporine	mean	5.91	1.01	1.53	0.19	5.13	1.00	1.49	0.42
		sem	0.03	0.01	0.06	0.04	0.05	0.00	0.06	0.04
1i	Go 7874	mean	5.77*	1.60	0.54	0.07	5.03*	1.91	0.66	0.24
		sem	0.16	0.17	0.04	0.00	0.16	0.08	0.00	0.11

			M_3				M_4			
					cooperativity				cooperativity	
name			pK	slope	^{3}H-NMS	ACh	pK	slope	^{3}H-NMS	ACh
1a	KT 5720	mean					6.42*	1.00	0.92	0.55
		sem					0.25	0.00	0.01	0.08
1b	K-252a	mean								
		sem								
1e	KT 5823	mean								
		sem								
1f	staurosporine	mean					5.31	1.00	0.88	0.15
		sem					0.09	0.00	0.02	0.03
1i	Go 7874	mean	5.12*	1.50	0.32	0.72	5.71*	1.47	0.80	0.10
		sem	0.01	0.50	0.09	0.05	0.13	0.21	0.05	0.00

Table 2

% inhibition of ^{3}H-NMS dissociation from muscarinic receptors by indolocarbazoles. Curves such as those shown in Figure 4 were fitted to a logistic equation as described in Mothods. The 'estd pK' is the product of affinity for the free receptor and cooperativity with ^{3}H-NMS derived from the equilibrium binding assays summarised in Table 1. The '*diff*' is the difference between the observed pK (-log IC_{50}) and 'estd pK'. Empty cells indicate that it was not possible to obtain at least 2 sets of parameter estimates. The ^{3}H-NMS dissociation rate constants (minutes^{-1}) observed in this study are (mean ± sem (n)): M_1 0.058 ± 0.002 (26); M_2 0.34 ± 0.01 (12); M_3 0.054 ± 0.002 (10); M_4 0.057 ± 0.002 (10).

			M_1					M_2				
name			pK	slope	Emax	estd pK	*diff*	pK	slope	Emax	estd pK	*diff*
1a	KT 5720	mean	6.18	1.00	56.79	6.70	*-0.52*				5.36	
		sem	0.15	0.00	3.81	0.09					0.26	
		n	7			6					5	
1b	K-252a	mean	5.54	1.40	100.00			5.65	1.00	35.05		
		sem	0.01	0.07	0.00			0.00	0.00	2.35		
		n	2					2				
1c	KT 5823	mean	6.40	1.00	103.55	6.58	*-0.18*	6.21	1.00	77.85	6.18	*0.02*
		sem	0.01	0.00	3.15	0.08		0.05	0.00	1.95	0.18	
		n	2			4		2			2	
1f	staurosporine	mean	6.01	1.00	104.73	6.10	*-0.09*	5.40	1.00	90.77	5.31	*0.09*
		sem	0.08	0.00	5.28	0.04		0.02	0.00	8.34	0.06	
		n	3			5		3			3	
1i	Go 7874	mean	5.70	1.34	100.00	5.50	*0.19*	5.30	1.31	100.00	4.85	*0.45*
		sem	0.02	0.01	0.00	0.19		0.01	0.01	0.00	0.16	
		n	2			2		2			2	

			M_3					M_4				
name			pK	slope	Emax	estd pK	*diff*	pK	slope	Emax	estd pK	*diff*
1a	KT 5720	mean	6.66	1.00	38.71			6.33	1.00	24.70	6.38	*-0.05*
		sem	0.15	0.00	1.52			0.08	0.00	0.21	0.24	
		n	3					3			2	
1b	K-252a	mean	5.55	1.00	37.10			5.37	1.00	48.95		
		sem	0.00	0.00	4.20			0.02	0.00	5.75		
		n	2					2				
1c	KT 5823	mean	6.45	1.00	29.80			5.88	1.00	41.50		
		sem	0.08	0.00	5.70			0.04	0.00	4.40		
		n	2					2				
1f	staurosporine	mean	5.48	1.00	67.10			5.61	1.00	88.00	5.25	*0.36*
		sem	0.07	0.00	2.77			0.08	0.00	6.20	0.08	
		n	3					3			3	
1i	Go 7874	mean	5.05	1.00	39.50	4.60	*0.45*	5.71	1.64	100.00	5.60	*0.11*
		sem	0.28	0.00	14.00	0.15		0.03	0.35	0.00	0.10	
		n	2			2		2			2	

Table 3

Parameter estimates from offrate assays. Emax indicates the maximal % inhibition of ^{3}H-NMS dissociation.
Empty cells indicate that at least two reliable estimates were not obtained. Estimates could not be obtained for 2f, 2g and 2i.
n>=3, except * n=2, # n=1

			M_1			M_2			M_3			M_4		
	Name		pK	slope	Emax	pK	slope	Emax	pK	slope	Emax	pK	slope	Emax
2a	987	mean	6.10	1	-91.90	5.81	1	-34.30	6.08	1	-146.35	6.04	1	-66.03
		sem	0.04	0	10.80	0.19	0	12.24	0.06	0	17.99	0.09	0	8.51
2b	WIN 51708	mean	5.55	1	49.71	5.93	1	94.66				6.78	1	95.78
	690	sem	0.12	0	3.51	0.02	0	1.16				0.06	0	4.01
2c	WIN 62577	mean	5	1	64.89	5.31	1.29	65.90				5.63	1	79.39
	691	sem	0.22	0	23.48	0.15	0.29	10.90				0.06	0	3.82
2d	924	mean	5.85	1.11	92.25	6.08	0.97	102.29	6.10*	1	29.79	6.98	1	101.79
		sem	0.05	0.12	4.36	0.03	0.03	2.26	0.12	0	6.09	0.03	0	3.12
2e	923	mean	5.32	1	88.52	5.58	0.99	93.12				6.19	1.02	97.40
		sem	0.10	0	1.74	0.01	0.01	3.26				0.04	0.01	1.37
2h	986	mean	4.68	1.77	101.67	4.34	1.62	100	3.50	1	100	4.63	1.37	99.63
		sem	0.04	0.08	1.67	0.09	0.09	0	0.02	0	0	0.08	0.12	0.37
2j	926	mean	4.71*	1.44	100	4.51*	1.02	101.10	4.72*	1	48.58	4.73*	1.53	87.83
cis		sem	0.08	0.09	0	0.05	0.02	1.10	0.01	0	3.64	0.12	0.53	15.37
2k	988	mean	6.86	0.99	101.02	6.21	1.06	94.52	6.71	1	11.21	6.30	1	91.50
		sem	0.04	0.01	1.46	0.05	0.06	1.99	0.16	0	1.47	0.07	0	3.24
2l	925	mean	4.77	1.79	99.78	4.84	1.68	103.67	4.60	1.77	100.77	4.78	1.81	100.91
		sem	0.02	0.08	0.22	0.01	0.07	1.88	0.02	0.06	0.77	0.04	0.07	1.59
2m	983	mean	5.17#	1	31.42	4.26#	1	105.20	4.92#	1	40.17	4.95#	1	90.54
		sem												
2n	982	mean				4.33	1	93.76	5.35	1	12.96	4.24	1.39	100
		sem				0.09	0	9.85	0.56	0	1.53	0	0.04	0

Table 4

Parameter estimates from equilibrium assays.
PK is the estimate of the log affinity of the agent for the free receptor.
'alloN' and 'alloA' are the estimates of cooperativity with ^{3}H-NMS and Ach respectively.
Estimates could not be obtained for 2f, 2g and 2i.
n>=3, except * n=2, # n=1

	Name		M_1 pK	M_1 slope	M_1 alloN	M_1 alloA	M_2 pK	M_2 slope	M_2 alloN	M_2 alloA	M_3 pK	M_3 slope	M_3 alloN	M_3 alloA	M_4 pK	M_4 slope	M_4 alloN	M_4 alloA
2a	987	mean	6.48	1.11	0.28	0.50	6.29	1.01	0.55	0.14	6.60	1.37	0.29	0.53	6.12	1.26	0.45	0.27
		sem	0.07	0.13	0.02	0.04	0.10	0.01	0.02	0.05	0.06	0.16	0.01	0.04	0.09	0.26	0.01	0.05
2b	WIN 51708	mean	5.77	1.24	0.25	0.44	5.93	1.22	1.70	0.22	5.46	1.33	0.46	0.74	6.18	1	3.12	0
	690	sem	0.07	0.14	0.04	0.07	0.06	0.22	0.21	0.01	0.13	0.33	0.09	0.17	0.03	0	0.25	0
2c	WIN 62577	mean	5.51	1	0.20	0.70	5.25	1	0.62	0.24	5.13	1.40	0.38	1.78	5.90	1	0.79	0.08
	691	sem	0.08	0	0.06	0.08	0.26	0	0.13	0.13	0.17	0.24	0.14	0.50	0.17	0	0.05	0.04
2d	924	mean	5.67	0.73	1.74	0.26	6.55	0.87	1.33	0.36	5.28	1	1.79	0.37	7.02	0.64	2.16	0.16
		sem	0.18	0.14	0.26	0.09	0.20	0.13	0.10	0.08	0.29	0	0.30	0.08	0.11	0.36	0.21	0.06
2e	923	mean	5.68	1	0.60	0.35	5.57	1	0.73	0.15	4.87	1	0.37	1.27	5.88	0.83	1.61	0.06
		sem	0.03	0	0.04	0.02	0.11	0	0.09	0.09	0.36	0	0.20	0.21	0.14	0.09	0.37	0.03
2h	988	mean	4.81*	1.33	0.26	0.15	4.79*	1.04	0.32	0.02	4.52*	1.42	0	0.12	4.99*	1.19	0.28	0.01
		sem	0.13	0.42	0.26	0.13	0.02	0.04	0.01	0.02	0.06	0.27	0	0.01	0.08	0.22	0.12	0.01
2j	926	mean	5.26*	1.23	0.04	0.18	5.27*	1.35	0.07	0.03	5.12*	1.58	0.02	0.23	5.52*	1.91	0.02	0
ds		sem	0.02	0.29	0.04	0.16	0.06	0.09	0.05	0.02	0.01	0.23	0.02	0.11	0.03	0.09	0	0
2k	988	mean	6.02	0.79	8.13	0.38	6.57	1.03	1.37	0.46	6.26	1	0.35	0.40	6.13	0.85	1.52	0.41
		sem	0.14	0.21	1.53	0.12	0.15	0.26	0.07	0.01	0.05	0	0.09	0.07	0.05	0.15	0.06	0.02
2l	925	mean	4.72*	2	0.79	0.08	4.50*	2.01	1.71	0.02	4.81*	1.66	0.60	0.02	4.62*	1.33	1.18	0
		sem	0.01	0	0.15	0.01	0.09	0.01	0.17	0.02	0.07	0.09	0.07	0.02	0.05	0.33	0.09	0
2m	983	mean	4.57#	1	0	0.23	4.96#	1	0.87	0.35	3.90#	1	0	0.20	4.57#	1	1.48	0.03
		sem																
2n	982	mean	4.31*	1	0	0.40	4.95*	1	1.02	0.56	4.13*	1	0.28	0.57	4.47*	1	0.76	0.12
		sem	0.09	0	0	0.09	0.36	0	0.03	0.08	0.22	0	0.28	0.05	0.12	0	0.09	0.12

Table 5

Comparison of affinity estimates at ^{3}H-NMS-occupied receptors from equilibrium (pKocc) and offrate (pKoff) assays. '*diff*' is the difference between pKocc and pKoff. n>=2

		M_1					M_2				
	Name	pKocc	sem	pKoff	sem	*diff*	pKocc	sem	pKoff	sem	*diff*
2a	987	5.92	0.09	6.10	0.04	*-0.18*	6.03	0.12	5.81	0.19	*0.22*
2b	WIN 51708	5.14	0.13	5.55	0.12	*-0.41*	6.15	0.06	5.93	0.02	*0.22*
2c	WIN 62577	4.63	0.37	5	0.22	*-0.37*	5.02	0.36	5.31	0.15	*-0.29*
2d	924	5.90	0.16	5.85	0.05	*0.05*	6.67	0.23	6.08	0.03	*0.59*
2e	923	5.46	0.05	5.32	0.10	*0.13*	5.43	0.17	5.58	0.01	*-0.16*
2h	986						4.29	0.04	4.34	0.09	*-0.05*
2j cis	926						3.97	0.32	4.51	0.05	*-0.55*
2k	988	6.91	0.06	6.86	0.04	*0.05*	6.70	0.12	6.21	0.05	*0.49*
2l	925	4.60	0.08	4.77	0.02	*-0.16*	4.73	0.05	4.84	0.01	*-0.11*
2n	982						4.96	0.35	4.33	0.09	*0.63*

		M_3					M_4				
	Name	pKocc	sem	pKoff	sem	*diff*	pKocc	sem	pKoff	sem	*diff*
2a	987	6.06	0.04	6.08	0.06	*-0.03*	5.77	0.09	6.04	0.09	*-0.27*
2b	WIN 51708						6.67	0.07	6.78	0.06	*-0.10*
2c	WIN 62577						5.80	0.18	5.63	0.06	*0.17*
2d	924	5.52	0.22	6.10	0.12	*-0.57*	7.35	0.15	6.98	0.03	*0.37*
2e	923						6.06	0.05	6.19	0.04	*-0.13*
2h	986						4.40	0.28	4.63	0.08	*-0.22*
2j cis	926						3.85	0.02	4.73	0.12	*-0.88*
2k	988	5.77	0.18	6.71	0.16	*-0.94*	6.31	0.06	6.30	0.07	*0.01*
2l	925	4.58	0.12	4.60	0.02	*-0.02*	4.69	0.09	4.78	0.04	*-0.09*
2n	982						4.34	0.17	4.24	0	*0.10*

Table 6

Estimates of log affinity values for the ^{3}H-NMS-occupied M_3 receptor and maximal % inhibition of ^{3}H-NMS dissociation rate, derived from competition assays with 2a analysed with the allosteric model (Equation 1 in Appendix 2). The cooperativity values were fixed at 0 (competitive or strong negative cooperative interaction) or 1 (neutral cooperativity, noninteracting). Slope values for the test agent were fixed at 1, except for staurosporine (slope=1.31 ± 0.16, n=3).

	Name	n	pK	Emax %	cooperativity
	gallamine	2	3.38 ± 0.02	100	1
	strychnine	4	4.06 ± 0.01	100	1
	KT 5720	3	6.11 ± 0.10	50 ± 4	0
	staurosporine	3	5.79 ± 0.17	43 ± 1	0
2b	WIN 51708	3	5.78 ± 0.06	0 ± 20	0
2k	988	2	no effect		

Claims:

1. A method for aiding in the identification of compounds capable of modulating the binding of a primary ligand to a muscarinic receptor by binding to an allosteric site of the muscarinic receptor which is capable of binding to compound 1a and/or compound 2a, the method comprising:

(a) contacting the muscarinic receptor and the primary ligand with one or more concentrations of a candidate compound; and,

(b) determining whether the candidate compound modulates the binding of a primary ligand to the muscarinic receptor by binding to the allosteric site of the receptor which is capable of binding compound 1a and/or compound 2a.

2. The method of claim 1 wherein brucine, gallamine or strychnine do not substantially bind to the allosteric site.

3. The method of claim 1 or claim 2, wherein the muscarinic receptor is a human M_1, M_2, M_3, M_4 or M_5 muscarinic receptor.

4. The method of any one of claims 1 to 3, wherein the candidate compound is selected if it enhances the binding of the primary ligand to the muscarinic receptor.

5. The method of any one of claims 1 to 4, wherein the candidate compound is selected if it reduces the binding of the primary ligand to the muscarinic receptor.

6. The method of claim any one of claims 1 to 5, wherein the method is repeated with different muscarinic receptor subtypes.

7. The method of claim 6, wherein candidate compound is selected if it binds the allosteric site and has no effect on the binding of the primary ligand to one or more of the muscarinic receptor subtypes but enhances or reduces the binding of the primary ligand at other muscarinic receptor subtype or subtypes.

8. The method of claim 1, wherein the candidate compound is selected if it changes the dissociation rate of the primary ligand from the muscarinic receptor or changes the ability of an allosteric ligand to affect the dissociation rate of the primary ligand from the muscarinic receptor.

9. The method of claim 8, wherein the allosteric ligand is capable of binding to the common allosteric site of the muscarinic receptor.

10. The method of any one of claims 1 to 9, wherein the binding of the candidate compound to the allosteric site is determined in assays employing two primary ligands which compete for the primary ligand binding site, one of which is labelled.

11. The method of claim 10, wherein the labelled primary ligand is NMS and the other primary ligand is ACh.

12. The method of any one of claims 1 to 11, wherein the method employs the muscarinic receptor, a candidate compound and a primary ligand, in the presence or absence of one or more concentrations of a further allosteric ligand.

13. The method of any one of claims 1 to 11, wherein the method determines the binding of a candidate compound to

the allosteric site using labelled primary ligand in assays which determine the primary ligand dissociation rate constant in the presence and absence of one or more concentrations of the candidate compound.

14. The method of claim 13, wherein the primary ligand is NMS.

15. The method of any one of claims 1 to 14, further comprising quantitating the effects of a test compound, which has been demonstrated in the general assays to be allosteric, on the equilibrium allosteric effects of ligands which are known to bind one or other of the two allosteric sites.

16. The method of any one of claims 1 to 15, wherein the primary ligand is acetylcholine (ACh) or N-methylscoplolamine (NMS), or another appropriate competitive muscarinic agonist or antagonist.

17. A method which comprises, having identified a candidate compound by the method of any one of claims 1 to 16, the further step of formulating the compound as a pharmaceutical composition.

18. Use of a compound as obtainable by the method of any one of claims 1 to 16 for the preparation of a medicament for the treatment of a conditions mediated by the binding of the primary ligand to the muscarinic receptor.

19. A method of modulating the response of a muscarinic receptor to a primary ligand, the method comprising contacting the muscarinic receptor with a compound which binds to an allosteric site of the muscarinic receptor which is capable of binding to compound 1a and/or 2a and

which thereby modulates the binding of the primary ligand to the muscarinic receptor.

20. Use of a compound for the preparation of a medicament for the treatment of a condition mediated by the binding of a primary ligand to a muscarinic receptor, wherein the compound binds to an allosteric site of the muscarinic receptor which is capable of binding to compound 1a and/or 2a and thereby modulates the binding of the primary ligand to the muscarinic receptor.

21. The use of claim 20, wherein the compound is represented by general formula 1 or 2.

22. The use of claim 20 or claim 21, wherein the primary ligand is acetylcholine (ACh) or N-methylscopolamine (NMS).

23. The use of any one of claims 20 to 22, wherein the condition is Alzheimer's disease, Parkinson's disease, motion sickness, Huntingdon's chorea, schizophrenia, depression, anxiety, sedation, analgesia, stroke, preanaesthetic, antispasmodic, irritable bowel syndrome, bladder-incontinence or retention, peptic ulcer disease, bronchitis/asthma/chronic obstructive airway disease, sinus bradycardia, cardiac pacemaker regulation, glaucoma, achalasia, symptomatic diffuse oesophageal spasm, biliary dyskinesia, scleroderma, diabetes mellitus, lower oesophageal incompetence, intestinal pseudo obstruction, regulation of sleep, control of pupil diameter or non-ulcer dyspepsia.

24. The use of any one of claims 20 to 23, wherein the binding of the compound to the allosteric site enhances the binding of the primary ligand to the muscarinic

receptor.

25. The use of any one of claims 20 to 23, wherein the binding of the compound to the allosteric site reduces the binding of the primary ligand to the muscarinic receptor.

26. The use of any one of claims 20 to 23, wherein the binding of the compound to the allosteric site has no effect on the binding of the primary ligand to one or more of the muscarinic receptor subtypes (neutral cooperativity) but has an allosteric effect (positive or negative cooperativity) at other subtype or subtypes.

27. The use of any one of claims 20 to 26, wherein the allosteric site does not bind brucine, gallamine or strychnine.

28. The use of any one of claims 20 to 27, wherein the muscarinic receptor is a human M_1, M_2, M_3, M_4 or M_5 muscarinic receptor.

29. Use of an allosteric site of a muscarinic receptor which is capable of binding to compound 1a and/or 2a in screening for compounds which are capable of modulating the binding of a primary ligand to a muscarinic receptor by binding to the allosteric site.

30. A compound represented by general formula 1 or 2 for use in a method of medical treatment.

31. The compound of claim 30, wherein the compound is an allosteric agent which modulates the binding of a primary ligand to a muscarinic acetylcholine receptor.

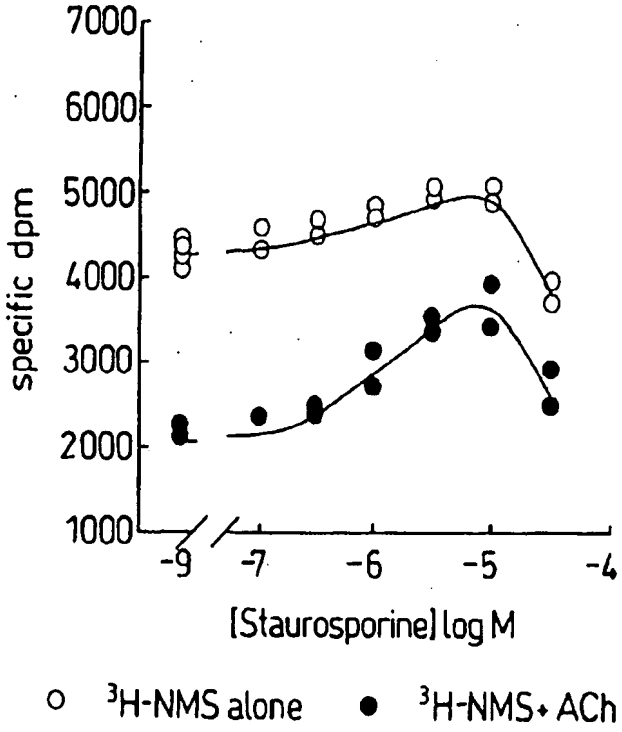

7000
6000
5000
4000
3000
2000
1000
specific dpm
-9
-7
-6
-5
-4
[Staurosporine] log M
^{3}H-NMS alone
^{3}H-NMS+ ACh

Fig. 1

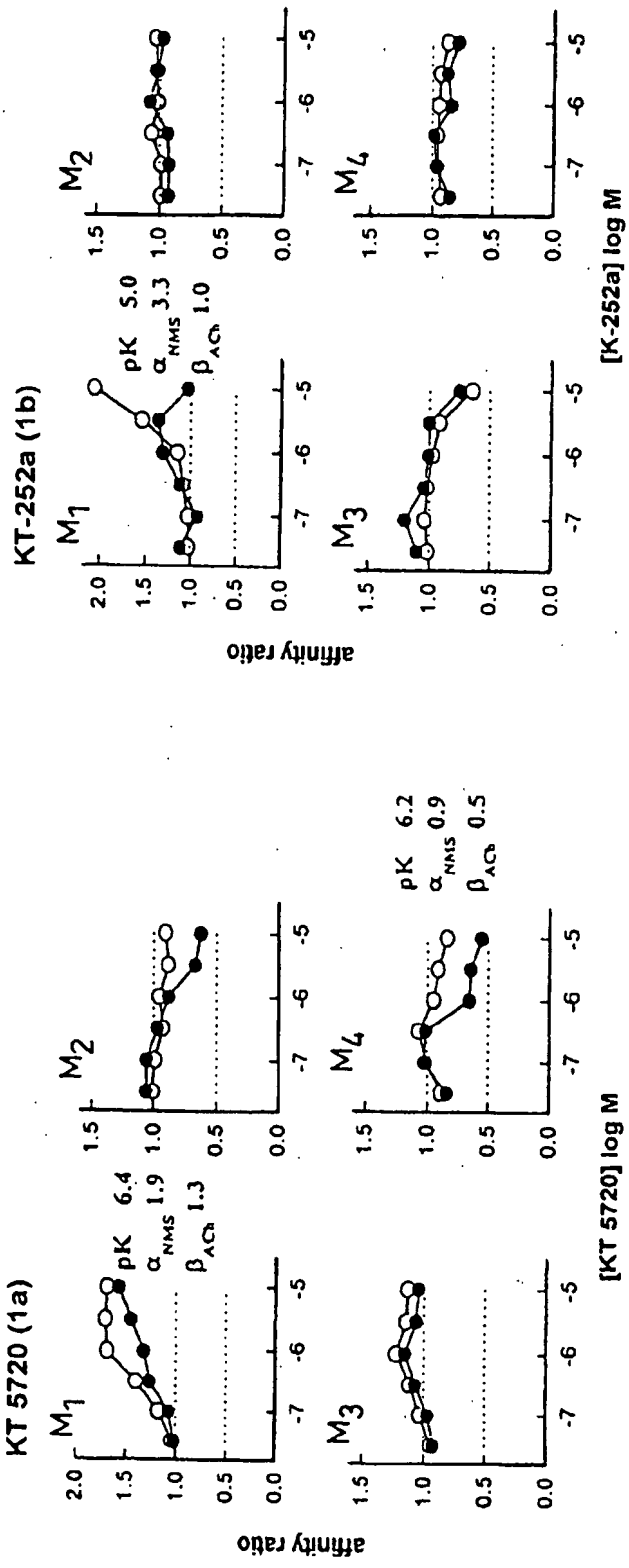


Fig. 2 (part 1 of 3)

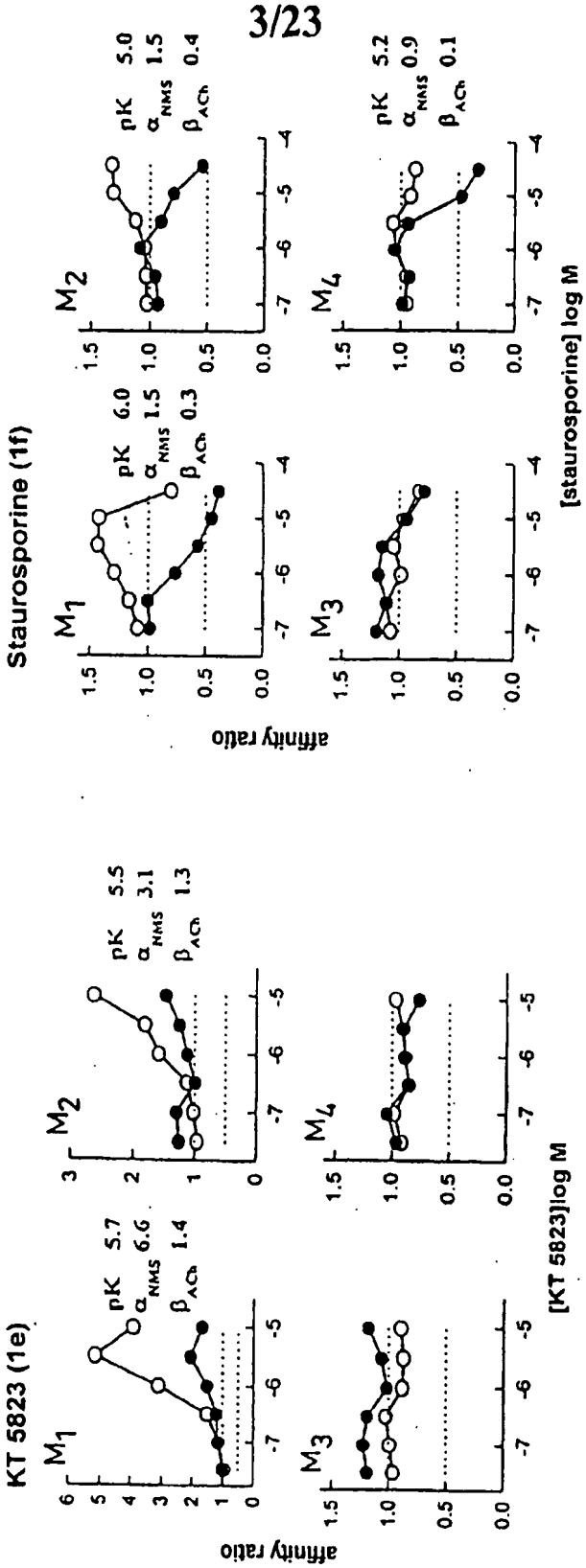


Fig. 2 (part 2 of 3)

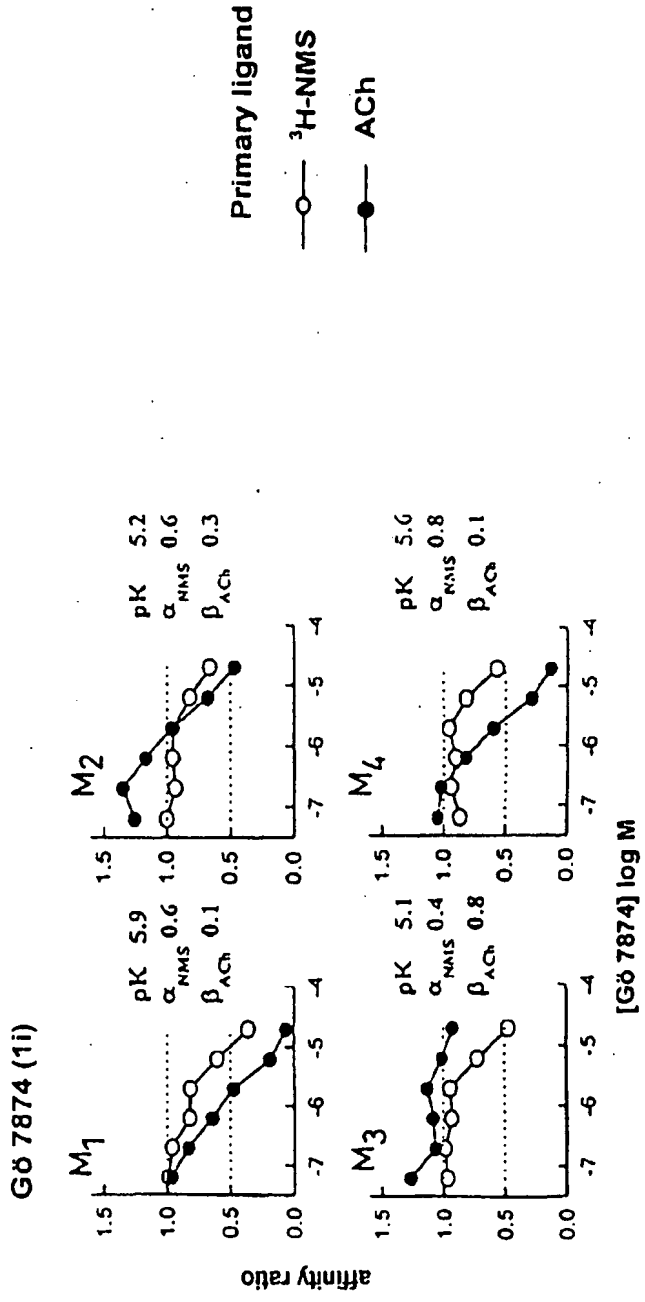


Fig. 2 (part 3 of 3)

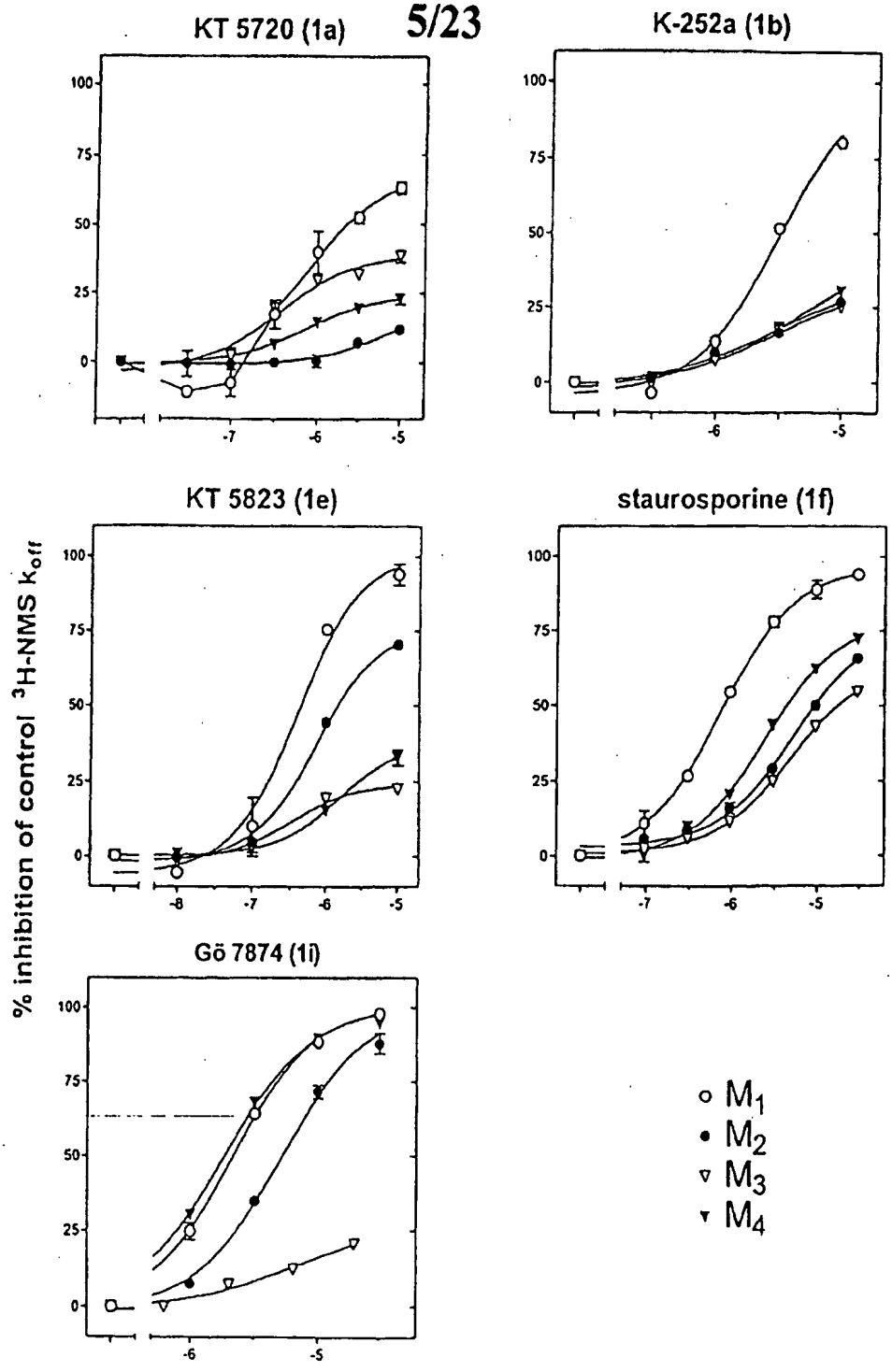

KT 5720 (1a)
K-252a (1b)
KT 5823 (1e)
staurosporine (1f)
Gö 7874 (1i)
% inhibition of control ^{3}H-NMS k_{off}
[agent] log M
M1
M2
M3
M4

Fig. 3

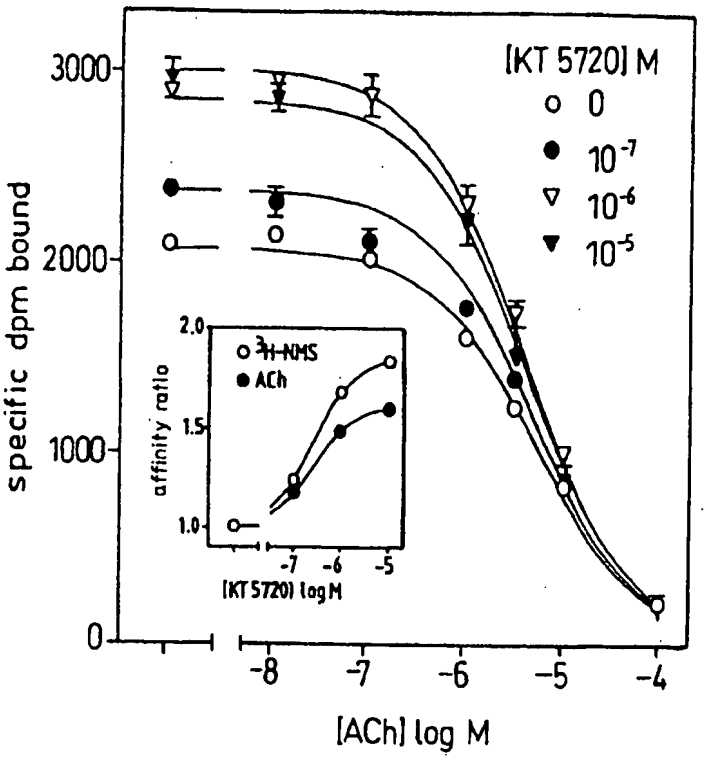

[KT 5720] M
0
10^{-7}
10^{-6}
10^{-5}
specific dpm bound
3000
2000
1000
0
-8
-7
-6
-5
-4
[ACh] log M
affinity ratio
2.0
1.5
1.0
^{3}H-NMS
ACh
-7
-6
-5
[KT 5720] log M

Fig. 4

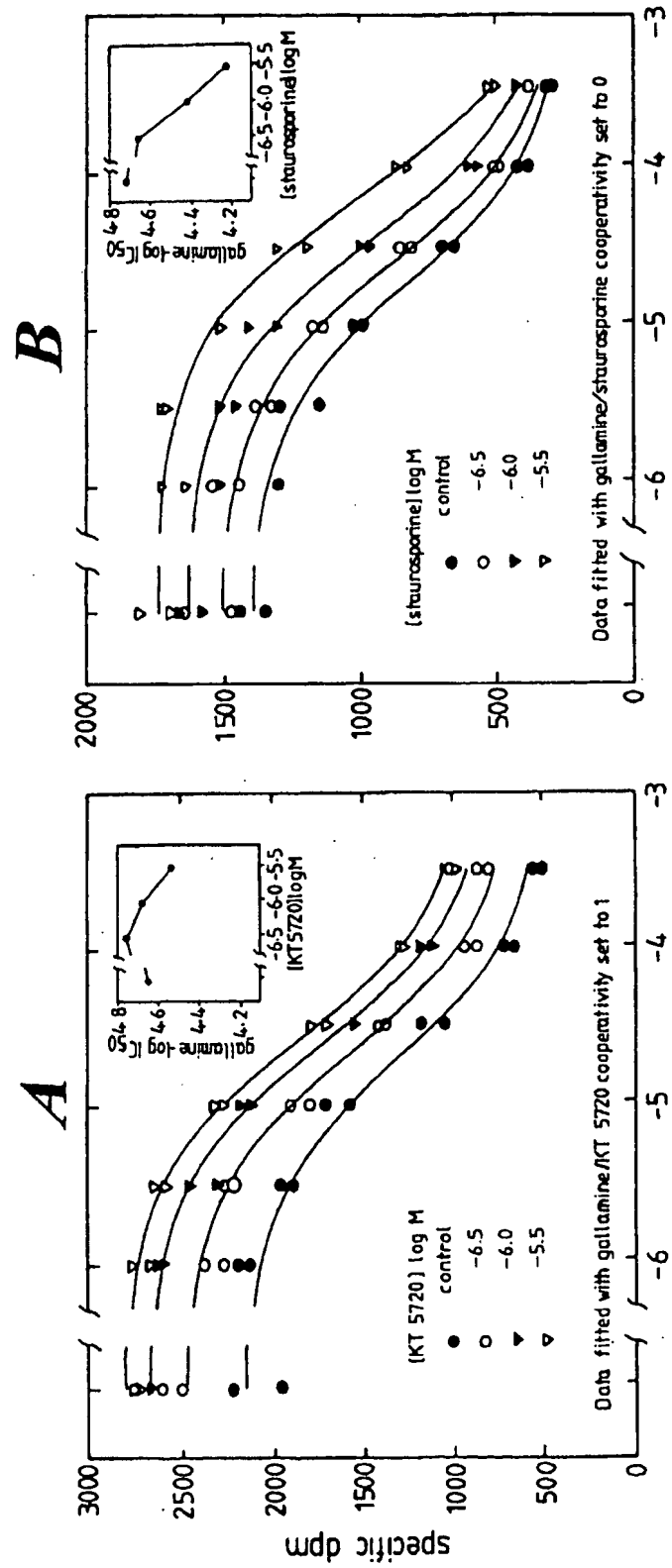

A
B
specific dpm
3000
2500
2000
1500
1000
500
0
-3
-4
-5
-6
[Gallamine] log M
(KT 5720) log M
control
-6.5
-6.0
-5.5
Data fitted with gallamine/KT 5720 cooperativity set to 1
gallamine -log IC50
4.2
4.4
4.6
4.8
-6.5 -6.0 -5.5
[KT5720]logM
(staurosporine) log M
control
-6.5
-6.0
-5.5
Data fitted with gallamine/staurosporine cooperativity set to 0
gallamine -log IC50
4.2
4.4
4.6
4.8
-6.5 -6.0 -5.5
[staurosporine]log M

Fig. 5

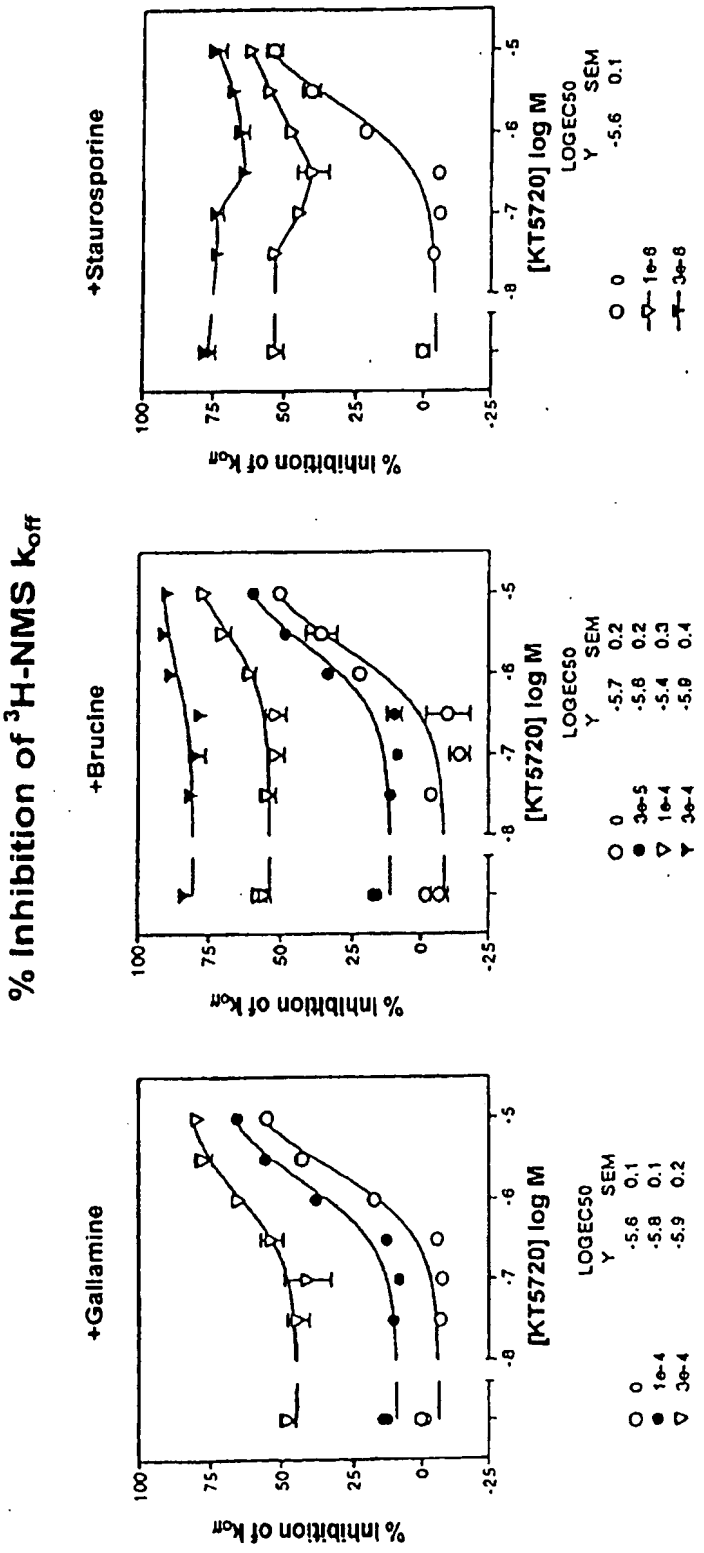


Fig. 6 (part 1 of 2)

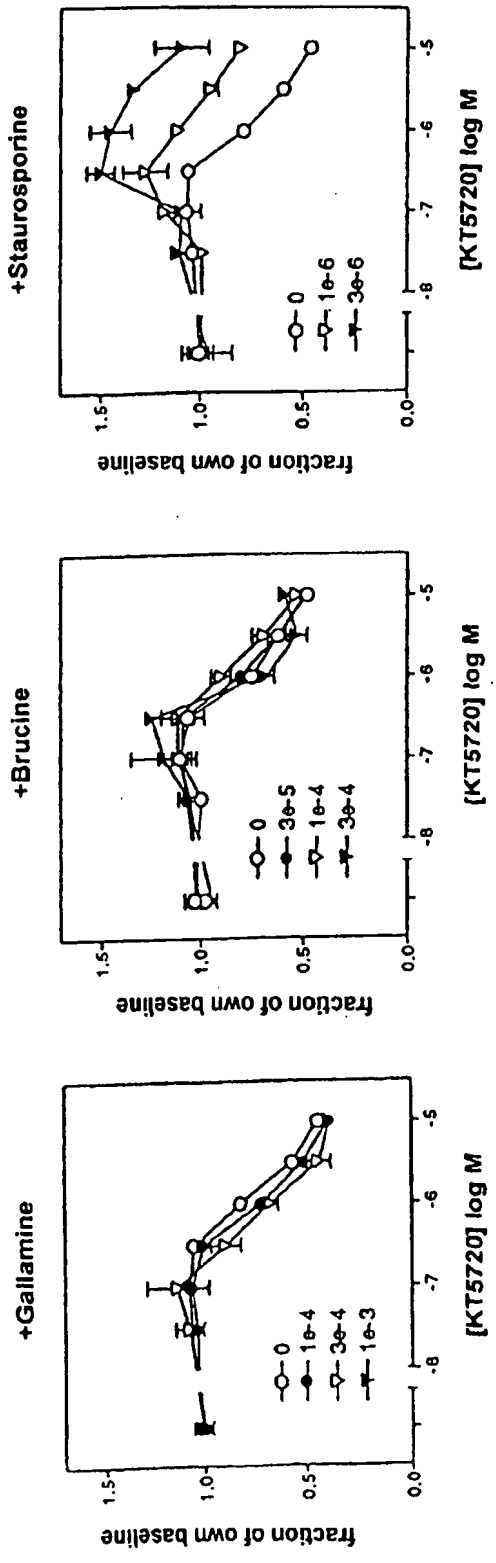


Fig. 6 (part 2 of 2)

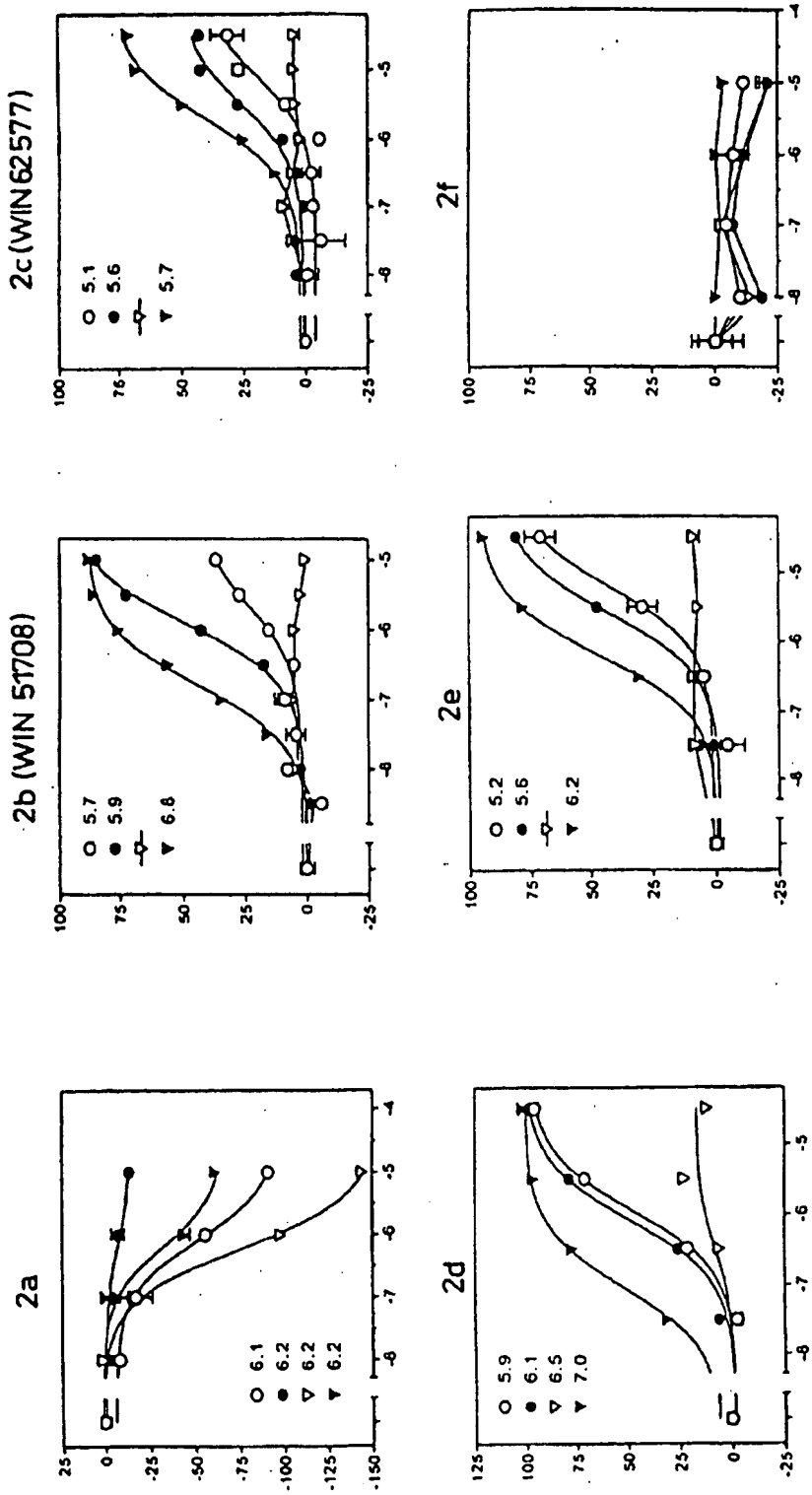
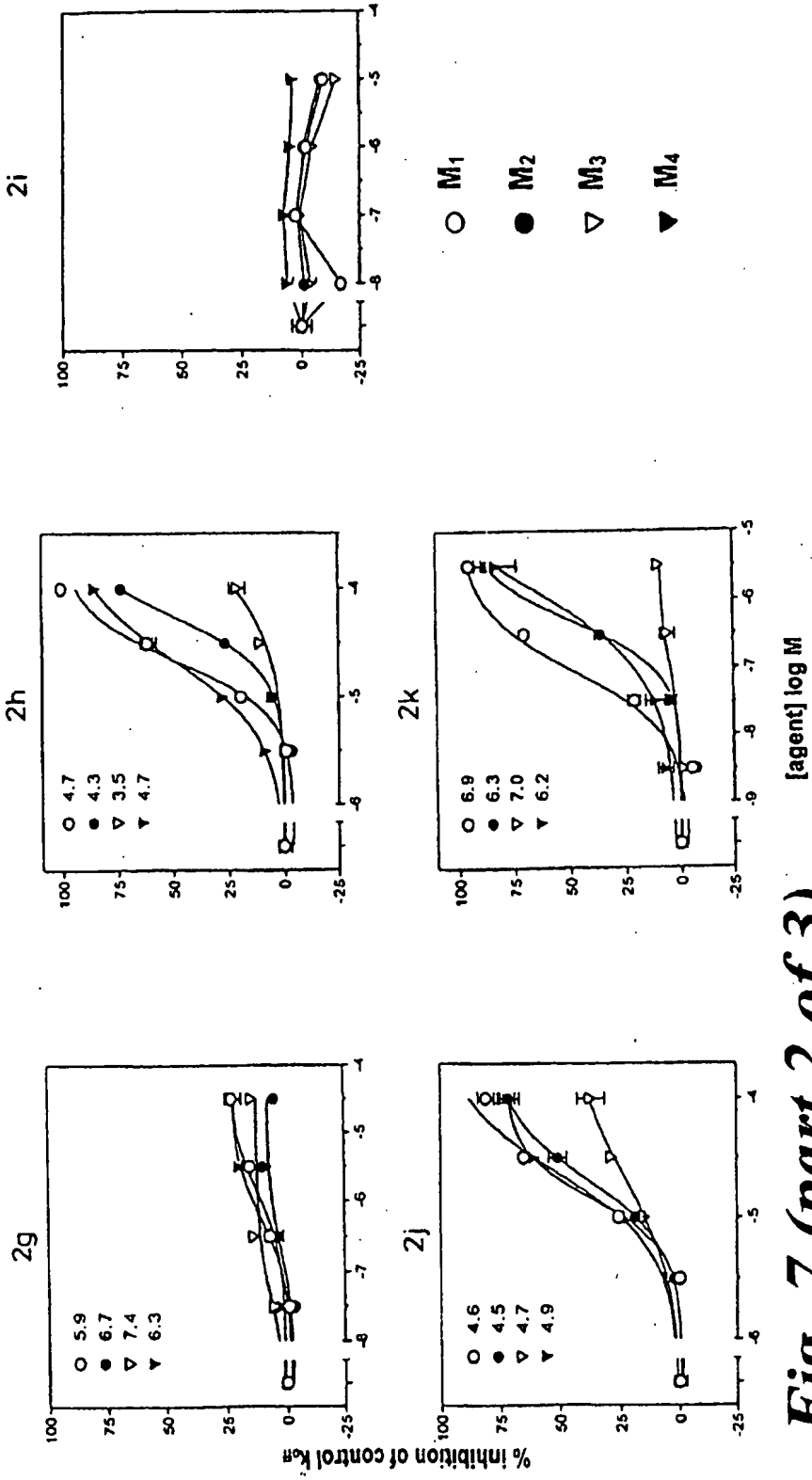


Fig. 7 (part 1 of 3)



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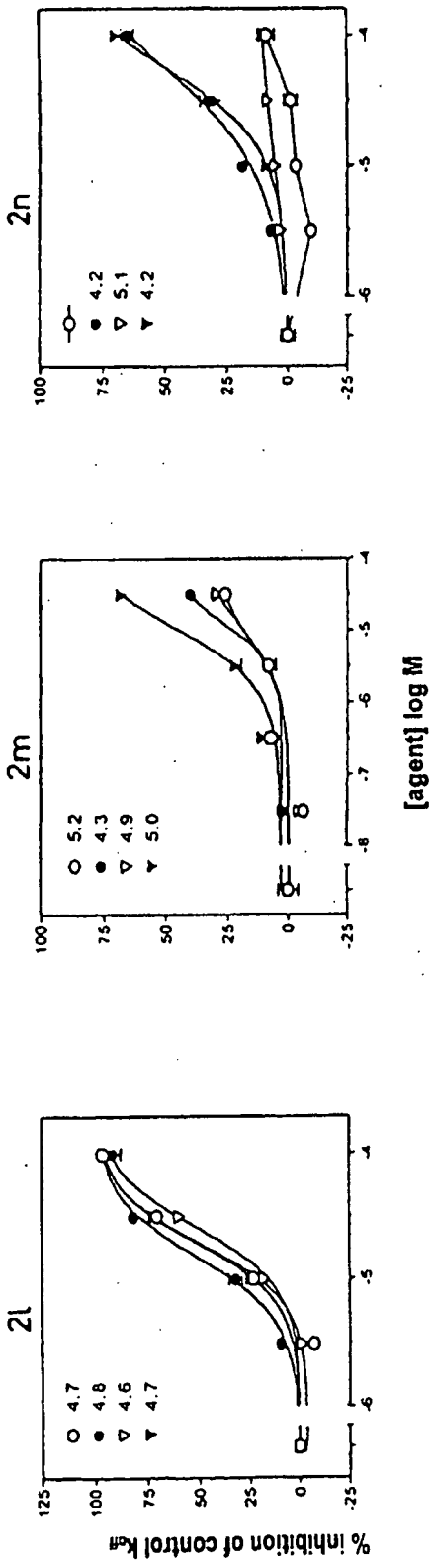

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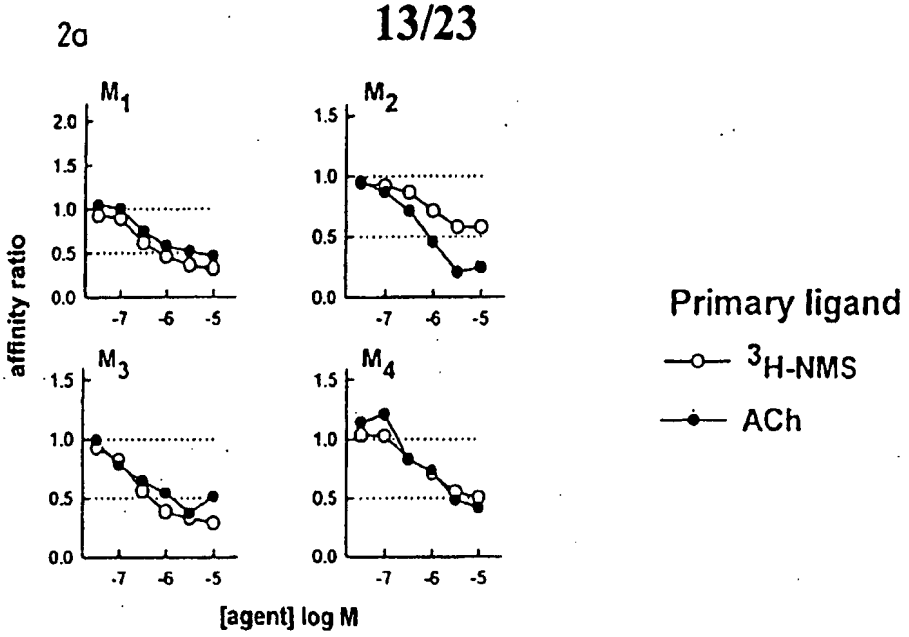


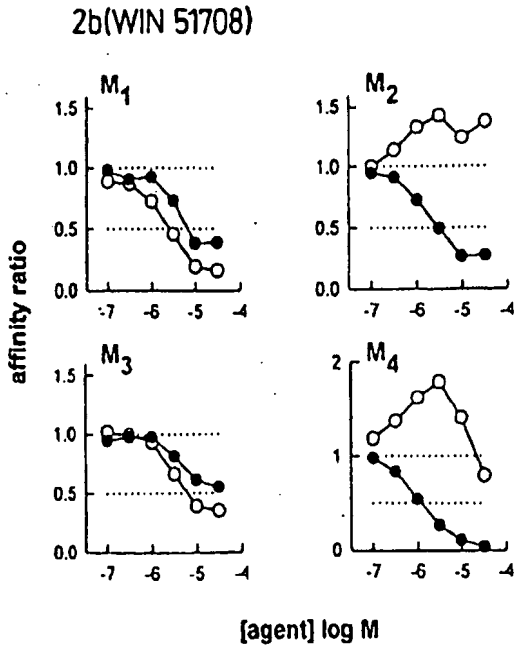
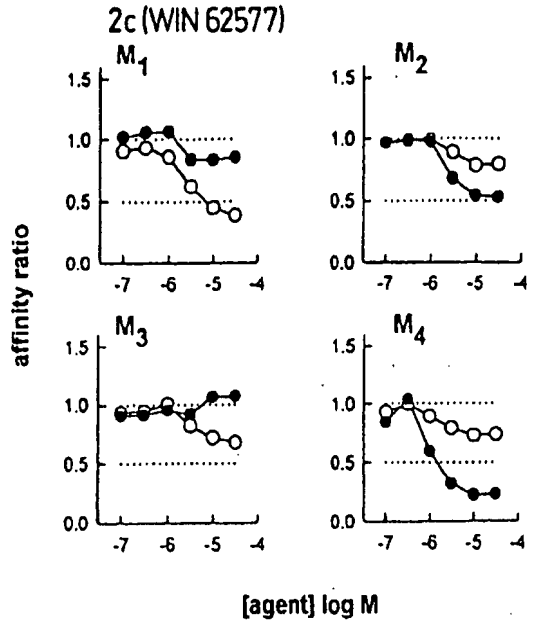


Fig. 8 (part 1 of 3)



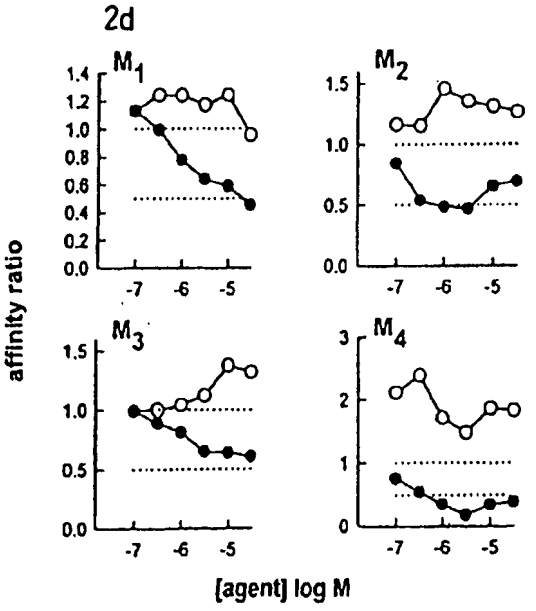


Fig. 8 (part 2 of 3)

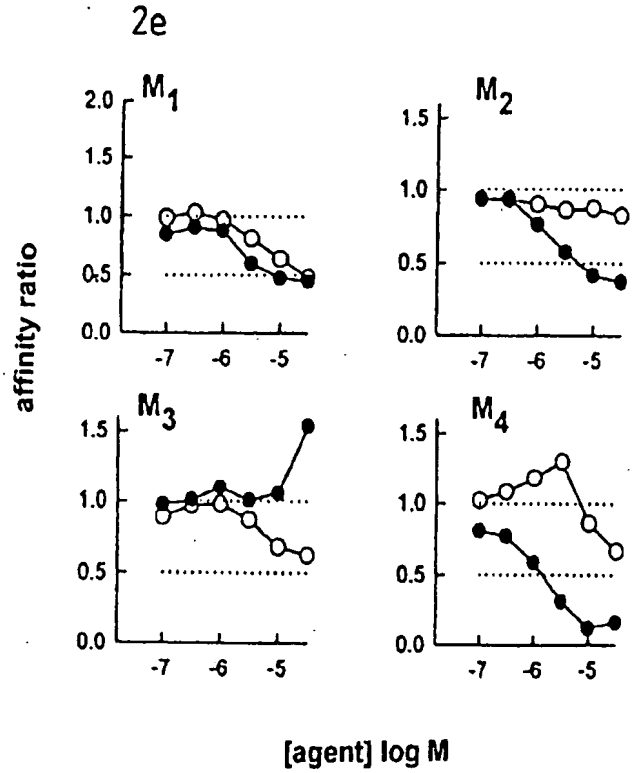
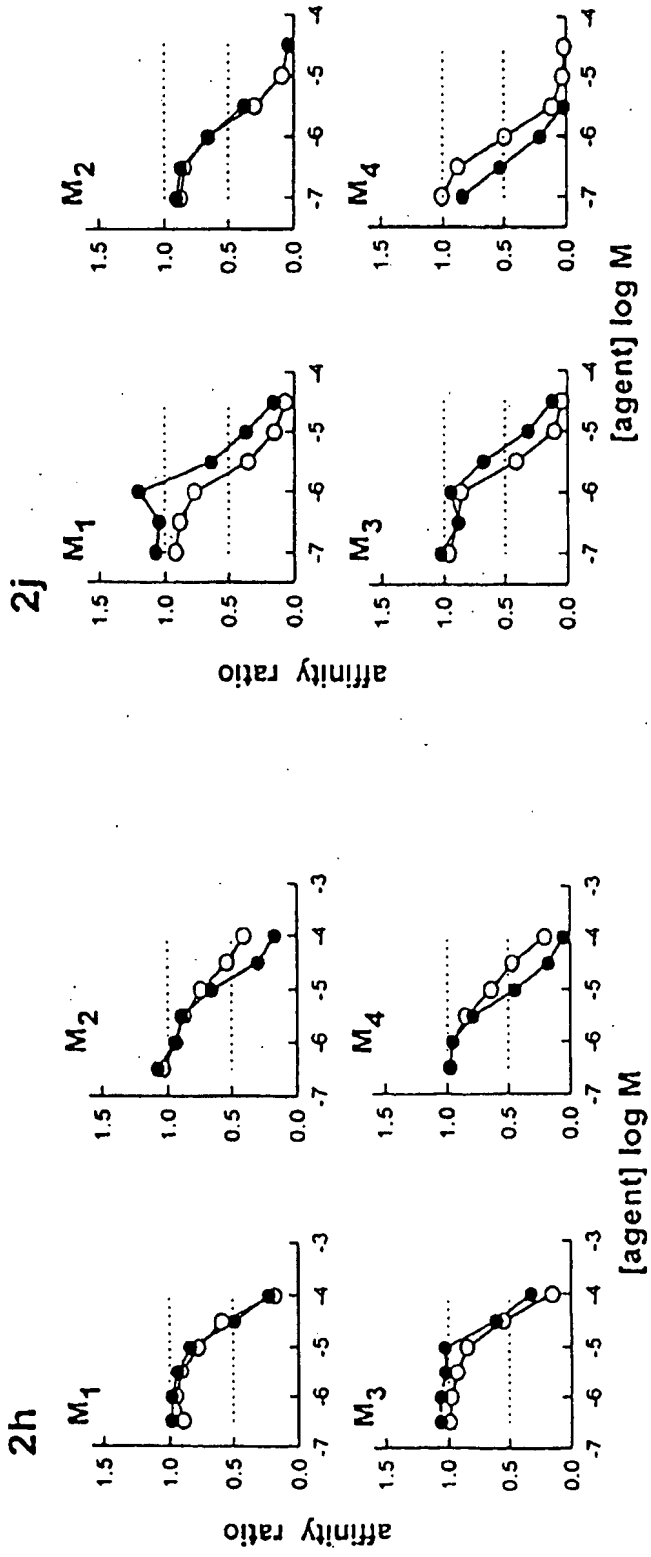


Fig. 8 (part 3 of 3)



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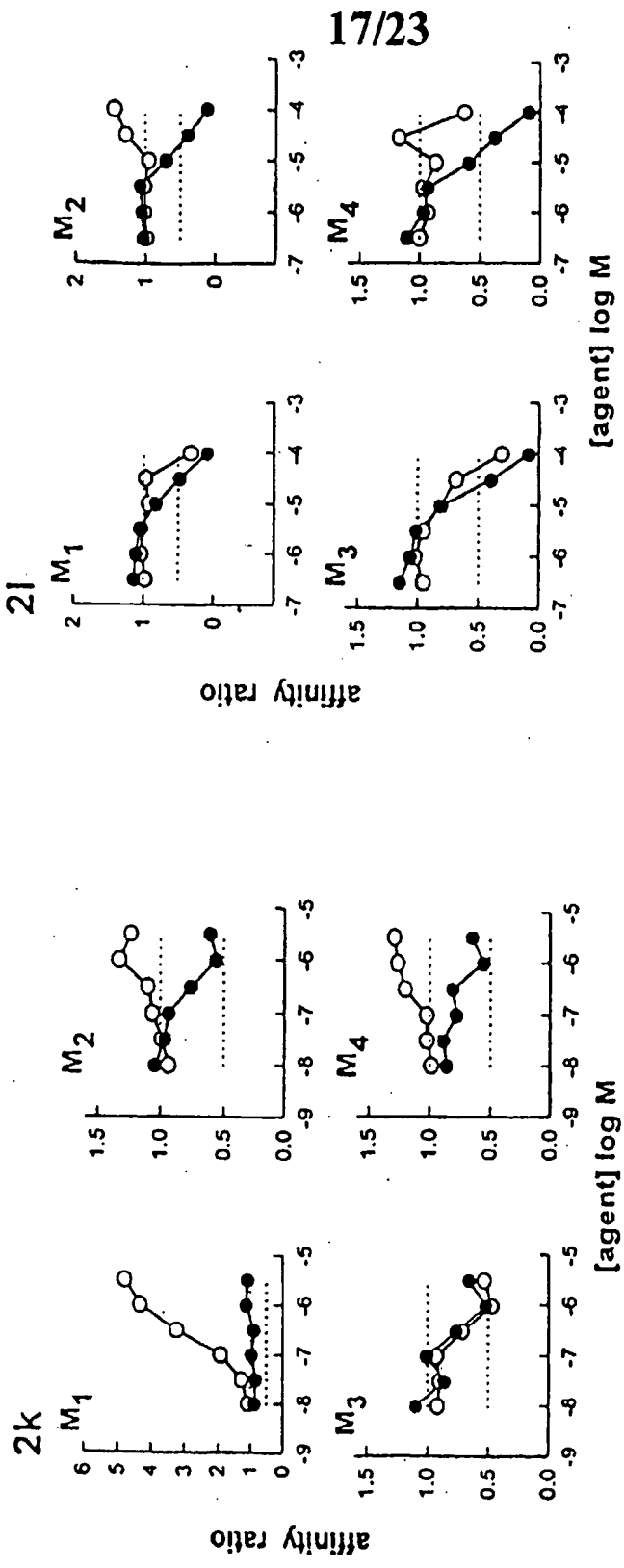


Fig. 9 (part 2 of 3)

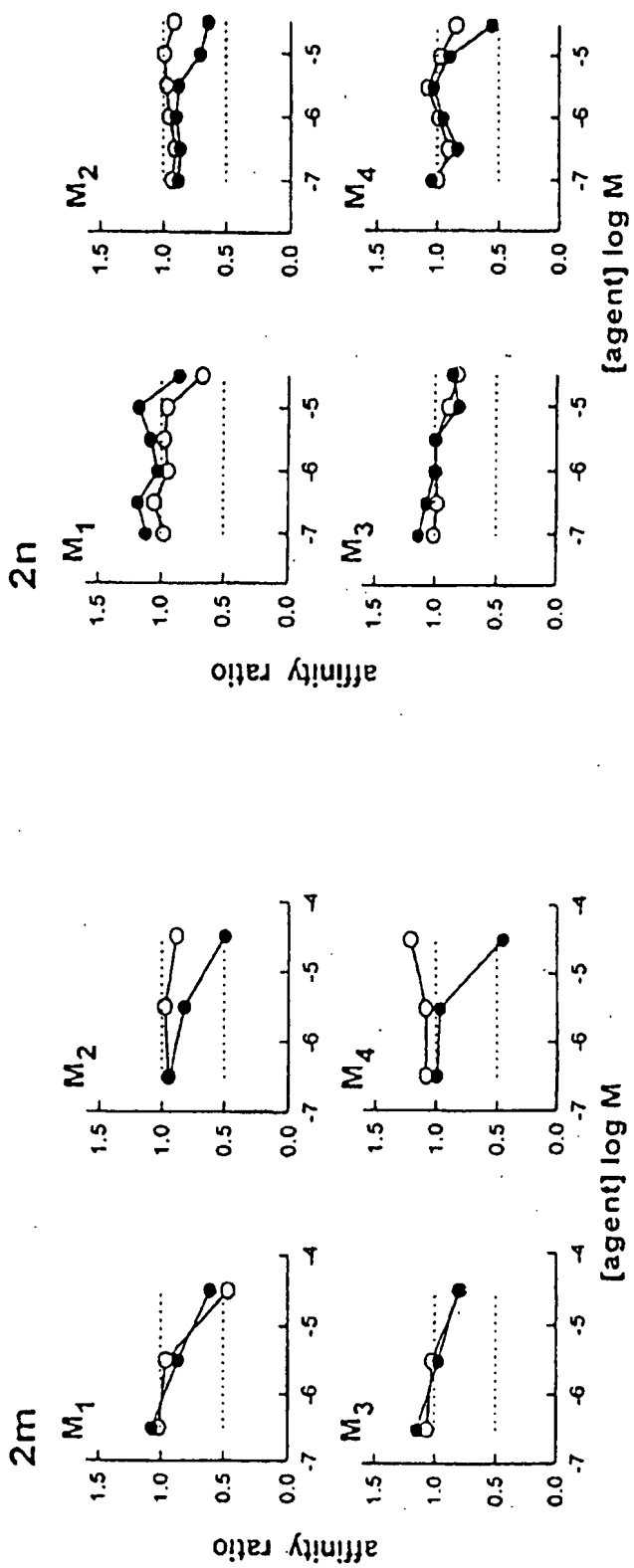


Fig. 9 (part 3 of 3)

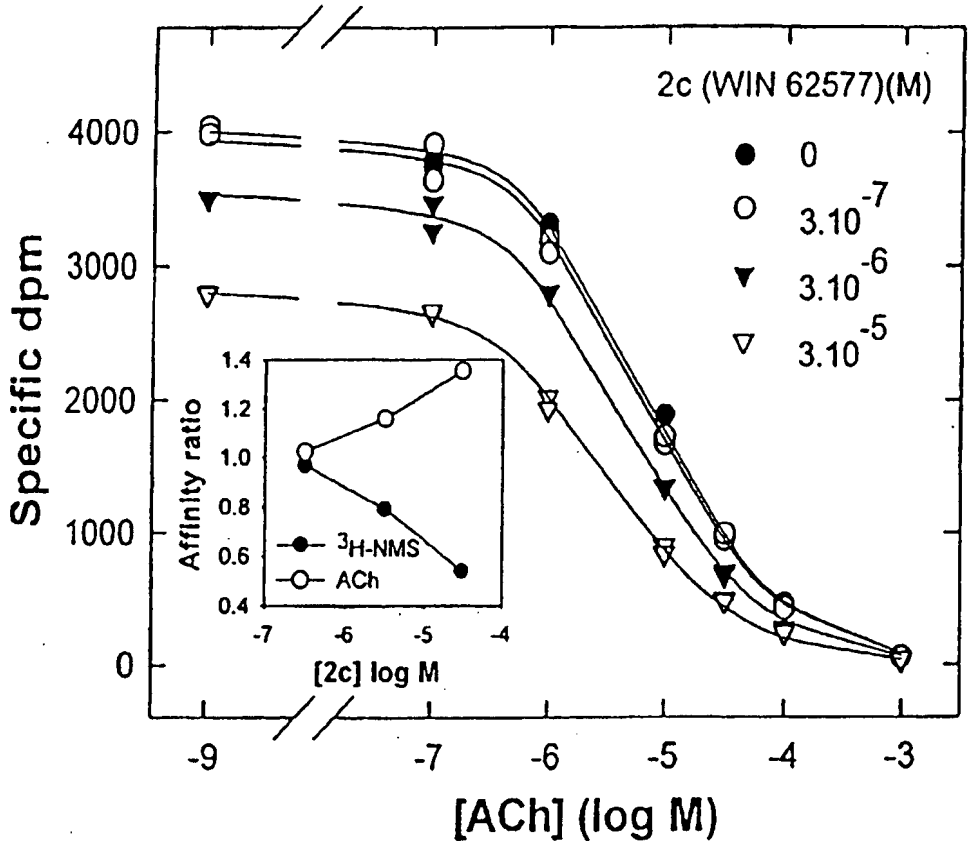

2c (WIN 62577)(M)
0
3.10-7
3.10-6
3.10-5
Specific dpm
4000
3000
2000
1000
0
-9
-7
-6
-5
-4
-3
[ACh] (log M)
Affinity ratio
1.4
1.2
1.0
0.8
0.6
0.4
-7
-6
-5
-4
[2c] log M
3H-NMS
ACh

Fig. 10

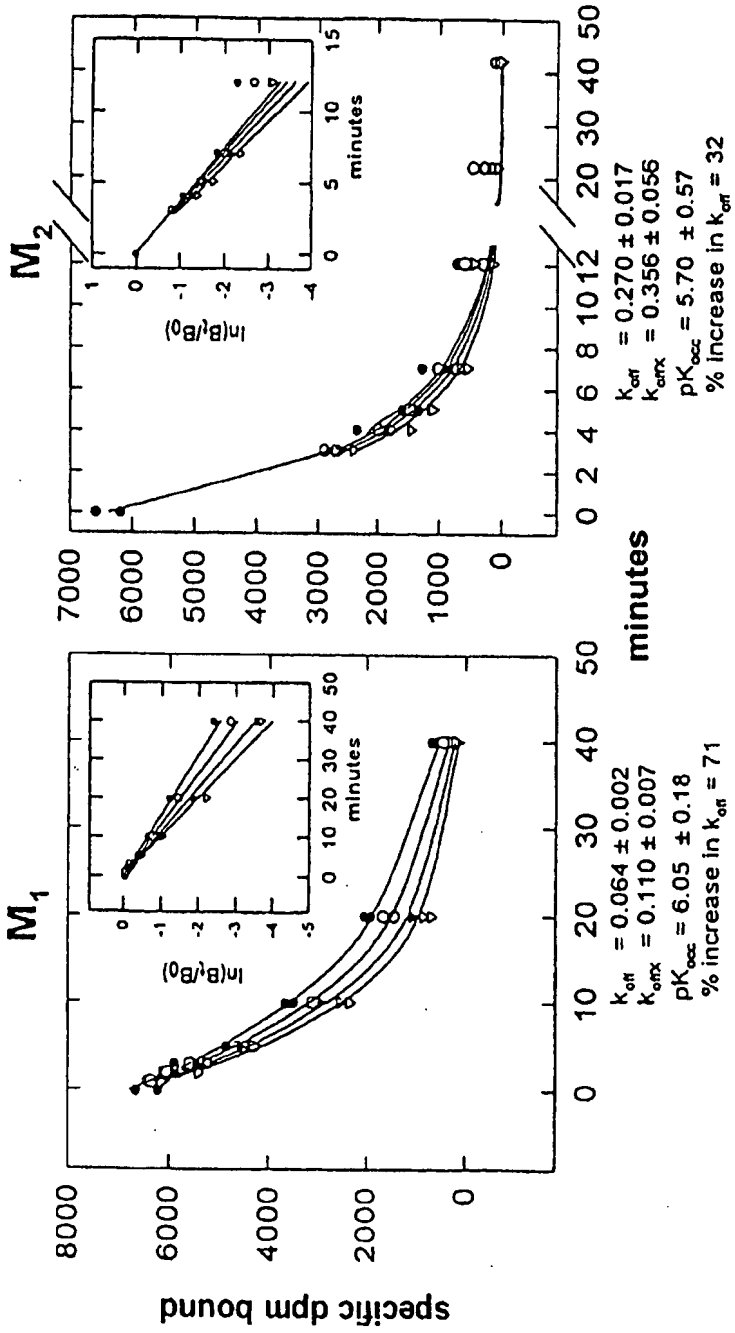


Fig. 11 (part 1 of 2)

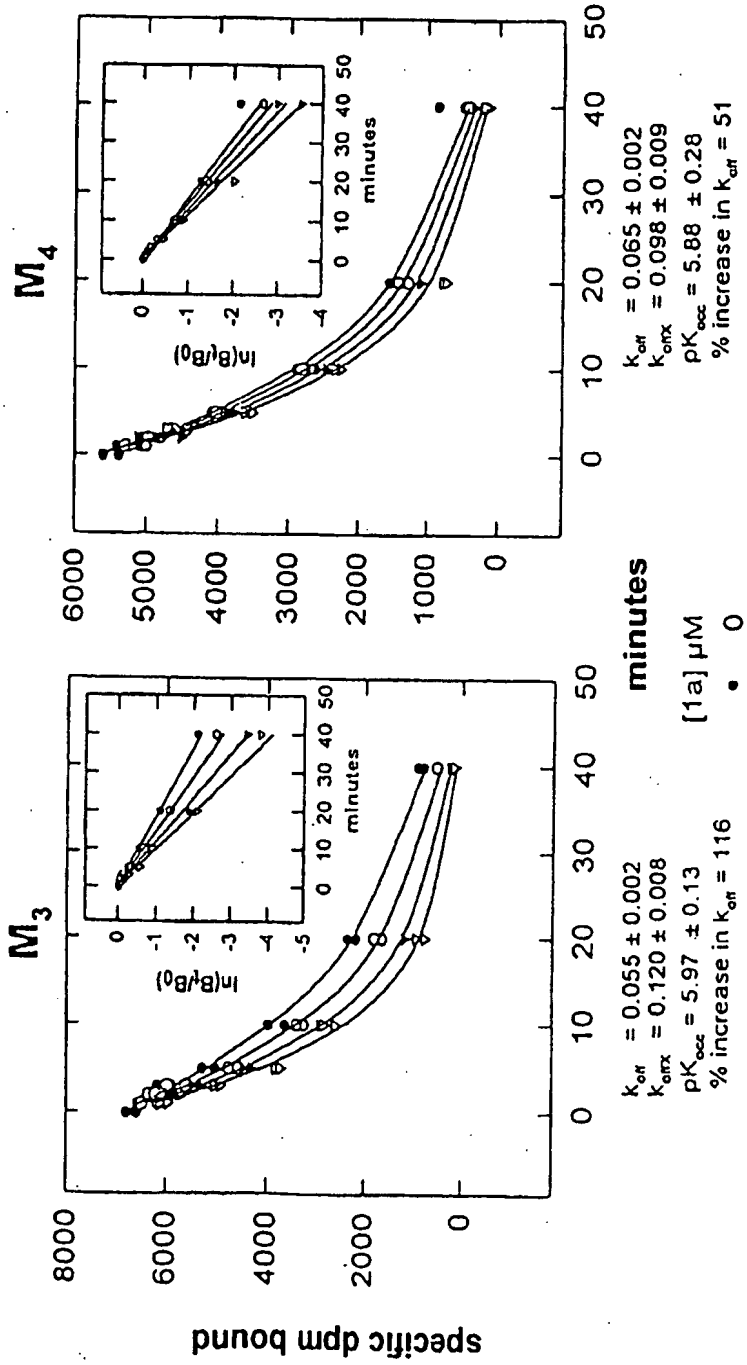


Fig. 11 (part 2 of 2)

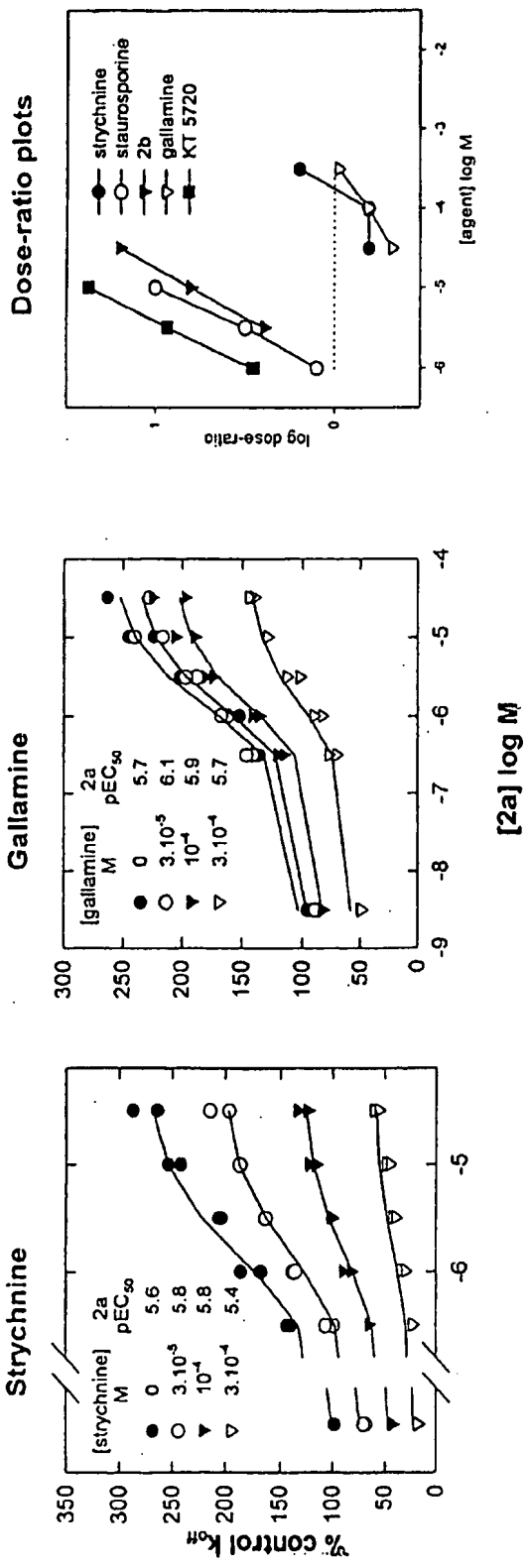


Fig. 12 (part 1 of 2)

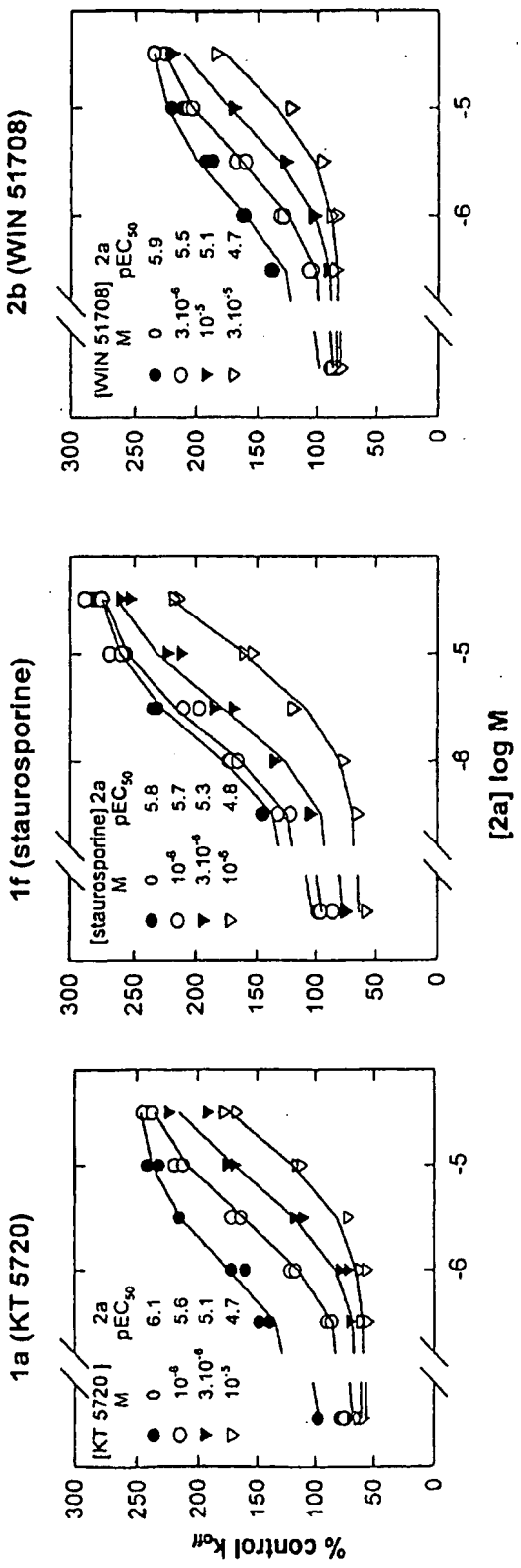


Fig. 12 (part 2 of 2)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 April 2001 (26.04.2001)

PCT

(10) International Publication Number
WO 01/29036 A3

(51) International Patent Classification[7]: G01N 33/566, A61K 31/395

(21) International Application Number: PCT/GB00/04064

(22) International Filing Date: 20 October 2000 (20.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9924962.5 21 October 1999 (21.10.1999) GB

(71) Applicant *(for all designated States except US)*: MEDICAL RESEARCH COUNCIL TECHNOLOGY [GB/GB]; 1-3 Burtonhole Lane, Mill Hill, London NW7 1AD (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BIRDSALL, Nigel [GB/GB]; 30 Oakleigh Park South, London N20 9JP (GB). LAZARENO, Sebastian [GB/GB]; 53 Crib Street, Ware, Hertfordshire SG12 9HF (GB).

(74) Agents: KIDDLE, Simon, J. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

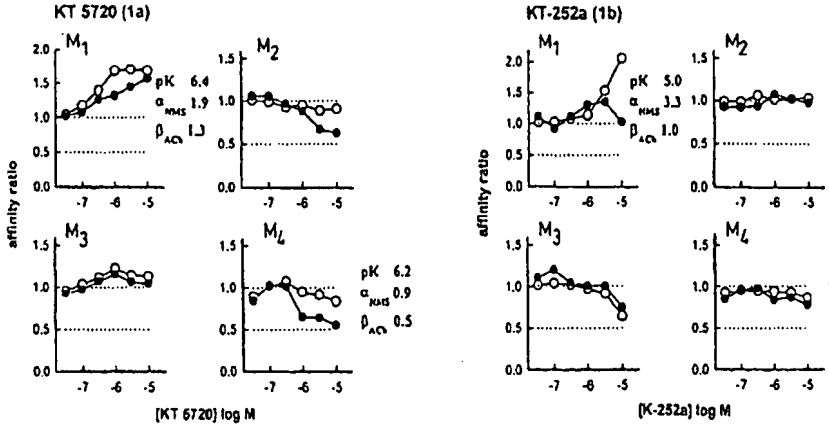
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
10 May 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ALLOSTERIC SITES ON MUSCARINIC RECEPTORS



(57) Abstract: An allosteric site on muscarinic receptors is disclosed, together with its use for screening for compounds capable of modulating the binding of a primary ligand such as acetylcholine to the receptor. The site is characterised herein a series of indolocarbazoles represented by formula (1) and a series of related compounds represented by formula (2). These compounds are capable of binding to the allosteric site to modulate the binding of a primary ligand to the receptors, showing positive, negative and neutral cooperativity and selectivity for muscarinic receptor subtypes.

WO 01/29036 A3

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 00/04064

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/566 A61K31/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2 292 685 A (SANKYO CO) 6 March 1996 (1996-03-06) cited in the application abstract --- WO 96 03377 A (BIRDSALL NIGEL ;LAZARENO SEBASTIAN (GB); SUGIMOTO MASAHIKO (JP); N) 8 February 1996 (1996-02-08) cited in the application abstract --- -/--	1-16,29
X		1-16,29

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 April 2001	12 07 2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hart-Davis, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ional Application No
PCT/GB 00/04064

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIRDSALL NIGEL J M ET AL: "Subtype-selective positive cooperative interactions between brucine analogs and acetylcholine at muscarinic receptors: Functional studies." MOLECULAR PHARMACOLOGY, vol. 55, no. 4, April 1999 (1999-04), pages 778-787, XP000997267 ISSN: 0026-895X cited in the application the whole document ---	1-16,29
X	LAZARENO S ET AL: "Subtype-selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: Radioligand binding studies." MOLECULAR PHARMACOLOGY, vol. 53, no. 3, March 1998 (1998-03), pages 573-589, XP000997226 ISSN: 0026-895X cited in the application the whole document ---	1-16,29
X	LAZARENO SEBASTIAN ET AL: "Detection, quantitation and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: Interactions of strychnine and acetylcholine at muscarinic receptors." MOLECULAR PHARMACOLOGY, vol. 48, no. 2, 1995, pages 362-378, XP000997227 ISSN: 0026-895X cited in the application the whole document ---	1-16,29
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1

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 00/04064

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LAZARENO SEBASTIAN ET AL: "Allosteric interactions of staurosporine and other indolocarbazoles with N-(methyl-3H)scopolamine and acetylcholine at muscarinic receptor subtypes: Identification of a second allosteric site." MOLECULAR PHARMACOLOGY, vol. 58, no. 1, July 2000 (2000-07), pages 194-206, XP000997198 ISSN: 0026-895X the whole document	1-16,29
A	US 5 747 336 A (BUCKLEY NOEL J ET AL) 5 May 1998 (1998-05-05) column 5, line 22 - line 64; table 1	1-16,29
A	US 5 677 327 A (WOLDEMUSSIE ELIZABETH ET AL) 14 October 1997 (1997-10-14) claim 1	1-16,29
A	WO 96 13506 A (KYOWA HAKKO KOGYO KK ;CEPHALON INC (US)) 9 May 1996 (1996-05-09) abstract page 2, line 8 - line 15	
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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

national application No.
PCT/GB 00/04064

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see further information sheet inevention 1

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-16, 29

   Methods for identifying compounds capable of modulating the binding of a primary ligand to a muscarinic receptor by binding to an allosteric site of the muscarinic receptor.

2. Claims: 17-28, 30-31 (all partially)

   Compounds for medical treatment, wherein the compounds are represented by the formulae 1a, 1b, 1c, 1d and 1e (page 55).

3. Claims: 17-28, 30-31 (all partially)

   Compounds for medical treatment, wherein the compounds are represented by the formula 1f (page 55).

4. Claims: 17-28, 30-31 (all partially)

   Compounds for medical treatment, wherein the compounds are represented by the formulae 1g, 1h and 1i (page 55).

5. Claims: 17-28, 30-31 (all partially)

   Compounds for medical treatment, wherein the compounds are represented by the formulae 2a, 2b, 2c, 2d, 2e, 2f, 2g, 2h, 2i, 2j, 2l, 2u (pages 56-57; see also general formula of page 4).

6. Claims: 17-20, 22-28 (all partially)

   Compounds for medical treatment, wherein the compounds are represented by the formulae 2k, 2m, 2n, 2o, 2p, 2q or 2r (pages 56-57).

7. Claims: 17-20, 22-28 (all partially)

   Compounds for medical treatment, wherein the compounds are represented by the formula 2s (page 57).

8. Claims: 17-20, 22-28 (all partially)

   Compounds for medical treatment, wherein the compounds are represented by the formula 2t (page 57).

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter onal Application No
PCT/GB 00/04064

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